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Some Pathologic Alterations of the Bovine Oviduct

LOUISE LOMBARD, D.V.M., Ph.D.; BANNER BILL MORGAN, Ph.D.;
SAMUEL H. McNUTT, D.V.M.

Madison, Wisconsin

LITTLE DETAILED information is available on the actual pathologic conditions that may be observed in the bovine oviduct. The practice of artificial insemination has emphasized the importance of lowered fertility and suggests an association with involvement of the oviduct in cattle.

The present study was made to determine the possible importance of tubal lesions and cyclic variations as causes of or factors contributing to repeat breeding in the cow. The various changes encountered in the oviducts are recorded in detail in this paper. Comparisons are made with the oviduct of the normal virgin heifer.[5,6]

LITERATURE

Considerable data in the literature indicate the extent of sterility which may be attributed to tubal lesions. Gilman[2] studied the oviducts of 86 cows, the majority of which were sterile, and presented case histories on 8 animals. In investigations of 211 cattle in Hungary, Küttel[3] found pathologic oviducts in 40 cases (18.9%). Of these, 6.6 per cent were due to miscellaneous infections, 3.3 per cent to tuberculosis, 1.9 per cent to hydrosalpinx, and 7.1 per cent to cysts within the mucosal folds.

Rowson[4] reported severe, bilateral adhesions of the oviduct in 13.3 per cent of 300 cows examined in the British Isles. Williams,[11] in America, estimated that tubal lesions were responsible for 15 per cent of the cases of sterility in cows. A similar estimate was made by Quinlan[7] for South Africa. Cembrowicz[1] studied a large series of animals and found pathologic oviducts in 2 per cent of 300 heifers and 8.9 per cent of 730 cows. Of these 1,030 animals, only 138 were examined both clinically and postmortem, the greater portion being examined by clinical methods only. The lesions recorded were hydrosalpinx, either unilateral or bilateral, salpingitis, adhesions, and cysts within the oviduct. According to Cembrowicz,[1] hydrosalpinx accounted for 1.5 per cent of the oviduct lesions (0.9% unilateral and 0.6% bilateral). Cysts on the inside of the oviduct were found in 1.3 per cent of the animals (only 0.3% were bilateral). Adhesions accounted for 2.5 per cent of the lesions.

MATERIALS AND METHODS

Studies were conducted on 154 repeat-breeding cows (71 Holstein-Friesians and 83 Guernseys). The selection of cows was based on: (1) at least one calving to exclude, as far as possible, congenital abnormalities preventing conception, (2) a minimum of four consecutive services without conception, thus excluding approximately 95 per cent of cows bred, (3) a maximum age limit of ten years which excluded infertility resulting from old age, (4) a maximum limit of 2 cows from any one herd, (5) rejection of cows with genital abnormalities as detected by rectal palpation, (6) exclusion of cows displaying purulent discharges, (7) regular occurrence of normal estrous cycles, and (8) a history of normal intervals between breedings.[10]

After several observations to ascertain the estrual cycle, each animal was inseminated. Cows were divided into two groups: those to be slaughtered and examined on the third day and those to be killed for study on the twentieth to thirty-fifth day postinsemination. The ova of the three-day cows were examined to determine whether fertilization had taken place. The oviduct on the ovulatory side was washed with physiologic saline in order to recover the ova. Fertilization was determined by the presence of 2 or more blastomeres as

From the Department of Veterinary Science, University of Wisconsin, Madison.

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Dr. Morgan was deceased Sept. 7, 1950.

observed at 440 magnifications. By the thirty-fourth day, the embryo was large enough for measurements and macroscopic observations, and the status of the embryo could be determined. The cows included in the thirty-four-day group which had returned to estrus were reinseminated and slaughtered three days later.

Sections were taken, whenever possible, from each of the three parts of both oviducts: isthmus, ampulla, and infundibulum. The tissues were fixed in the following solutions: calcium cadmium formol,[4] Mossman's, Helly's, and mercury bichloride formol. Paraffin sections were cut longitudinally at 7 μ and stained with Weigert's iron and modified Crossman's triple stain, Giemsa's, and 1 per cent thionin.

FINDINGS AMONG THREE-DAY REPEAT BREEDER COWS

Sixteen cases of major pathologic alterations were observed among 103 cases of three-day repeat breeder cows (table 1).

TABLE 1—Pathologic Alterations in the Bovine Oviduct of 154 Repeat Breeders

Time post-insemination examined	Total No.	Pyosalpinx	Chronic interstitial salpingitis	Hydrosalpinx	Per cent of total
3 days	103	3.0	5.0	8.0	...
Per cent of each	...	2.9	4.8	7.8	15.5
20-35 days	51	2.0	4.0	0.0	...
Per cent of each	...	4.0	8.0	0.0	12.0

They consisted of 8 cases of hydrosalpinx, 3 cases of pyosalpinx, and 5 cases of chronic interstitial salpingitis. In addition, adhesion of the fimbriae to the ovary was detected in 7 cases. None was considered severe.

Of the remaining 80 cases, the general architecture of the serosal and muscular coats did not vary noticeably from that found in the normal virgin heifer.

The mucosal epithelium in 23 (22%) of the 103 cows closely resembled that seen in the virgin heifer during the period from five days postestrus to one day proestrus.[5,6] Among 39 per cent which contained homogeneous globules and cytoplasmic projections, the protruding epithelial cells were absent. Homogeneous globules were found in the mucosal epithelium of 75 (73%) of the 103 animals. Protruding epithelial cells were present in 29 cases (28%), and cytoplasmic projections in 101 (98%) of the cases. The mucosal epithelium of only 1 case (H45, group 1) lacked evidence of secretory activity.

Mucosal cysts, varying from spherical to oval, were observed in the lamina propria just below the basement membrane of the epithelium (fig. 1). The cysts ranged in size from 30 by 140 μ to 30 by 155 μ and were lined with a low cuboidal to columnar epithelium. These cysts were found in 26 cases (25%). Similar cysts were not observed in the oviducts of the virgin heifers. Lymph follicles were seen in the lamina propria of cows H69, G37, and H20 in group 2.

Cysts were observed on the serosa in 9 cases. In no instance did they produce a narrowing of the lumen. The cysts varied in size from 7 by 9 mm. to 15 by 30 mm. In 3 cases, the cysts were parovarian and adjacent to the ampulla. The cysts were lined by a squamous to a low cuboidal epithelium which lay upon a coat of connective tissue. In 6 cases, hydatid cysts of Morgagni were noted at the borders of the fimbriae and, in these cases, a muscular wall was noted to surround the cuboidal epithelial lining. In 55 of the 103 (53%), serosal nodules were observed. (For an explanation of the terms employed in the foregoing, reference is made to the report of Lombard.[5])

DEFINITE PATHOLOGIC ALTERATIONS

Of the major alterations in the oviduct of the series of 103 three-day repeat breeder cows, 3 were pyosalpinx, 8 hydrosalpinx, and 5 chronic interstitial salpingitis. Two of the 3 cases of pyosalpinx were unilateral and the other bilateral. The entire wall of the oviduct was infiltrated with polymorphonuclear leucocytes, lymphocytes, and plasma cells (fig. 2). In cows H40 and H13 (group 1), the majority of cells were of the lymphocytic type. In cow G10, the predominant cell was the neutrophil. The infiltration was most pronounced in the lamina propria. The lumen was almost entirely, if not completely, obstructed by cellular exudate. The mucosal epithelium contained large numbers of cytoplasmic projections similar to those seen in the postestrual phase of the normal virgin oviduct.

Three of the 8 cases of hydrosalpinx were bilateral and 5 were unilateral. In 1 of the unilateral cases, the lesions in-

Fig. 1 — Longitudinal section through the ampullary portion of the oviduct from cow 52 (group 1) of the three-day repeat breeder cows. Two mucosal cysts may be seen in the lamina propria.

volved a portion of the wall but did not completely obstruct the lumen. Nevertheless, the lesions offered an obstruction to the passage of the ovum, as indicated by an entrapped ovum. The cases of hydrosalpinx were not infiltrated by any cells.

The microscopic picture seen in hydrosalpinx was that of a number of chambers (fig. 3) varying in size and shape, lined by a squamous or low cuboidal type of epithelium (fig. 4). The chambers contained a light-staining, granular material. Cells and debris lay free in these chambers. Cilia were not seen on the epithelial cells lining these cysts. The condition did not always involve the entire tubular portion of the oviduct. In 6 cases, only the ampulla was involved.

Of the 5 cases of chronic interstitial salpingitis, 3 were bilateral and 2 were unilateral. The condition was characterized by extensive infiltration of all the

Fig. 2 — Pyosalpinx. Longitudinal section through the oviduct of three-day repeat breeder cow H13 (group 1).

tubal layers, especially of the mucosa, with lymphocytes and considerable numbers of plasma cells. The mucosal epithelium was infiltrated in numerous places by lymphocytes. Two cases (H77 and H43, group 2) had accompanying adhesions between the fimbriae and the ovary. Three (G93, H43, and H77, group 2) had serosal nodules.

FINDINGS AMONG TWENTY-DAY TO THIRTY-FIVE-DAY REPEAT BREEDER COWS

Fifty-one cows of the twenty- to thirty-five-day repeat breeder group revealed major pathologic changes, including 2 cases of pyosalpinx and 4 cases of chronic interstitial salpingitis (table 1). All cases of pyosalpinx and chronic interstitial salpingitis were bilateral. The description of both conditions was included with that of the three-day repeat breeder cows. Adhesions were detected in 2 cases.

The mucosal epithelium in 27 (53%) of the cases resembled the epithelium seen in virgin heifers during the period extending from five to fifteen days postestrus. In this respect, they were similar to the findings in 9 pregnant cows previously described.[5] Homogeneous globules were observed in 42 cases (82%), protruding epithelial cells in 32 cases (63%), and cytoplasmic projections in 45 cases (98%).

Mucosal cysts, similar to those described among the three-day repeat breeder cows, were seen in 19 cases (37%).

Increased numbers of eosinophils, neutrophils, lymphocytes, and plasma cells were present, as compared with the averages from the virgin heifers.

In 20 (39%) of the 51 cases, serosal nodules similar to those described for the virgin heifer were found on the serosa. Three hydatid cysts of Morgagni were noted on the fimbriae of the oviduct of cow G100.

DISCUSSION

The mucosal epithelium of the three-day repeat breeder cows generally resembled the epithelium seen in the virgin heifers during the period of five days postestrus to one day proestrus. Cytoplasmic projections were the most consistent finding and were plentiful even in cases of pyosalpinx. Homogeneous globules in the mucosal epithelium were next in frequency. Possibly, one or more of these forms of secretion may function in the early nourishment of the embryo.

Mucosal cysts in the lamina propria were described by Küttel,[3] who found a 7.1 per cent incidence among 211 cows examined. The prevalence of cysts in our series was much higher (25%). In animals H69, G36, and H20, lymph follicles were found in the

Fig. 3 — Hydrosalpinx. Cross section through the oviduct of three-day repeat breeder cow H10 (group 1). The mucosal folds are absent and thin dividing partitions split the lumen into numerous locules.

uterine horns, in addition to those in the oviduct. Lymph follicles were more commonly found in the uterus (approximately 16% of this series of 103 cows) than in the oviduct (approximately 2%).

Correlation between increased leucocyte counts in the oviduct and uterus was generally poor but, in 2 cases with high eosinophilic numbers in the oviduct (H23 and G10, group 1), similar high counts were obtained in the uterus.

Major pathologic changes were observed in 15.5 per cent of the animals. Of these, 2.9 per cent were pyosalpinx; 4.8 per cent of the cases represented chronic interstitial salpingitis, and 7.8 per cent, hydrosalpinx. In only 3 cases could the findings be correlated with significant observations on the uterus. In cows H4 and H44 of group 1, chronic interstitial metritis accompanied a similar disturbance of the oviduct. Cow H13 of group 1 manifested pyometra in addition to the bilateral pyosalpinx.

The percentage of major pathologic alterations in this series is comparable with that of Williams.[11] The incidence of adhesions obtained in this series (8.7%) is much higher than the 2.5 per cent observed by Cembrowicz[1] and is about half the 13.3 per cent recorded by Rowson.[9]

An attempt was made to correlate viability and anomalies of the ovum with alterations seen in the oviducts of the 103 cows. In 3 cases of bilateral hydrosalpinx (H7, H10, group 1, and H85, group 2), ova were recovered from 2 cases (H7 and H85) but not in the third (H10). The ovum had been fertilized in H85. The entire wall of the oviduct was not involved on the ovulatory side, thus allowing passage of the sperm and ovum. In H7, the ovum was infertile. The ova from 2 of 5 cases of unilateral hydrosalpinx were recovered (G43 and H19, group 1). However, in H19, the ovum was infertile. Ova were not recovered from G46 (group 2), H4, and H8 (group 1). The oviduct involved in hydrosalpinx affords some explanation of these findings since, in all 3 cows, hydrosalpinx existed on the ovulatory side. In case H19, an infertile ovum was recovered above the involved area.

An ovum was recovered from each of the 2 cases of unilateral pyosalpinx (H40 and H13, group 1). The ovum of H40 was infertile, that of H13 fertile and normal in appearance. Pyosalpinx in both H40 and H13 was on the nonovulatory side. An ovum was not recovered from G10, a case of bilateral pyosalpinx.

Ova were not recovered from 4 (H44, G93, H77, and H43) of 5 cases of chronic interstitial salpingitis. An infertile ovum was obtained from G36. Ovulation did not occur in case H77. Cows G93 and H43 had bilateral chronic salpingitis. However, H43

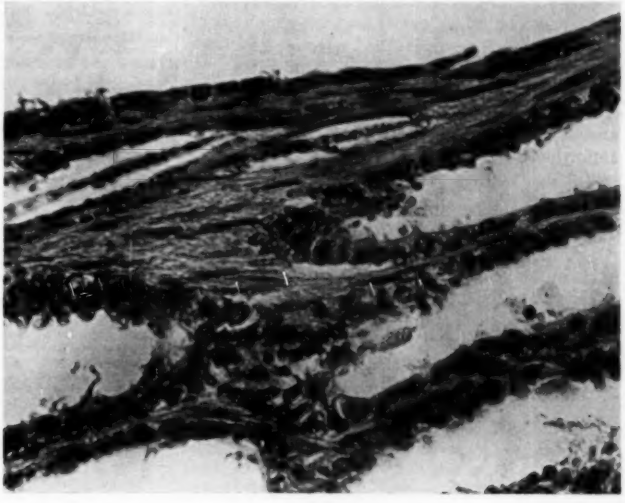

Fig. 4 — Hydrosalpinx. Cross section through the oviduct of three-day repeat breeder cow H10 (group 1). Note the flat cuboidal to low columnar epithelium and a few cytoplasmic projections from the epithelium.

also had adhesions of the fimbriae to the ovary, though not apparently severe enough to hinder passage of the ovum. Cow H44 had unilateral chronic salpingitis on the ovulatory side, while in G36 there was bilateral involvement.

The mucosal epithelium in the twenty- to thirty-five-day repeat breeder cows resembled closely that of virgin heifers during the period of five to fifteen days postestrus, as well as that of the 9 pregnant cows studied.[5] As in the three-day repeat breeder cows, cytoplasmic projections, followed by homogeneous globules, were the most consistent finding in the mucosal epithelium. Compared with the three-day repeat breeder cows, the tissues of the twenty- to thirty-five-day repeat breeder cows showed an increase in the relative amounts of secretion, especially of the homogeneous globules.

Major pathologic alterations were detected in 12 per cent of the 51 cows of the twenty- to thirty-five-day series. Of these lesions, 4 per cent were pyosalpinx and 8 per cent were chronic interstitial salpingitis. Pyosalpinx in 2 of these cases (H16, group 1 and G47, group 2) was associated with a purulent metritis. One case of chronic interstitial salpingitis (G37, group 2) was associated with pyometra.

The percentage of serosal nodules was somewhat less in the twenty- to thirty-five-day cows than among the three-day cows. The nodules were found in cow H17 (group 1), a case of pyosalpinx; and in cows G38, G28 (group 1), and H94 (group 2), all cases of chronic interstitial salpingitis.

All the major lesions (12%) were associated with the failures to recover embryos and with 2 of the 7 cases in which dead embryos were found. Other possible contributing factors which were studied apparently did not influence the development of the embryo.

SUMMARY

Gross and pathologic observations were made on the oviducts of 154 repeat breeder cows. Of 103 cows in a three-day repeat breeding group, 16 showed major pathologic alterations which included 8 cases of hydrosalpinx, 3 of pyosalpinx, and 5 of chronic interstitial salpingitis. Cysts on the serosa were observed in 9 cases. Serosal nodules were observed in 55 of the 103 animals.

Of 51 cows representing twenty- to thirty-five-day repeat breeders, 6 showed major pathologic changes including 2 cases of pyosalpinx and 4 of chronic interstitial salpingitis. Serosal nodules were observed in 20 of the cases. Three hydatid cysts of Morgagni were detected on the fimbriae of 1 heifer.

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Capillary Tube Method for Conducting the Brucella-Stained Antigen Milk Test

NELSON B. KING, D.V.M., M.Sc.

Columbus, Ohio

THE PREVENTION and control of brucellosis in man is largely dependent upon the control and eradication of the disease in domestic animals.

Brucellosis in cattle is routinely detected by means of the standard serum-agglutination test and, in the final analysis, this test is the basis of the entire brucellosis control and eradication program in this country. The test has been shown by continued usage to be sufficiently reliable to warrant disposal of animals showing a blood serum titer of 1 : 100 or higher. In the majority of cases, these animals have been found to be infected. Hence, interstate regulations and control programs have been based on this test.

Brucellosis investigators and livestock disease control officials, while recognizing the merits of the blood serum-agglutination test, realize that its practicability leaves much to be desired. It is thought that the spread of Brucella infection in domestic animals is in direct proportion to the movement of livestock in the interval between tests.

During and following World War II, the movement of cattle in this country had increased tremendously. The interval between tests for brucellosis had increased, hence, the incidence of the disease in cattle has more than doubled in some areas, with an average rise throughout the United States. This condition may be largely due to the expense and impracticability of the blood serum-agglutination test when used as the principal diagnostic tool.

A simpler method, which may find practical application in the detection of Brucella-infected herds, has recently come into use in this country. The test is known as the *Brucella abortus* ring test in the United States and, in Europe, as the Abortus Bang Ring (ABR) test. Its use promises to ease the struggle of dairy farmers in their efforts to locate, control, and eliminate brucellosis from their herds.

One of the principal limitations of the present method of using the ring test is that it has not been as efficient when used on milk samples of individual animals as when used on pooled herd samples. Most investigators feel that this is probably caused by the lack of cream or variation in the cream-rising capacity of the milk of the individual animals.

Approximately, one and one-half hours at room temperature is required before sufficient normal creaming takes place in order for a reading to be made. Obviously, this restricts sampling to milk which creams normally, and limits the number of tests which may be conducted in a given time.

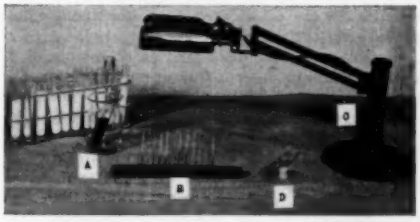

Fig. 1—Equipment used in conducting capillary tube test. A, antigen; B, plasticine; C, magnifying glass and light; and D, capillary tubes in vial.

The desirability of a rapid, economical, and practical method of diagnosing brucellosis by testing milk from individual animals is recognized. With this view in mind, the following work is presented.

Review of Literature

The literature shows that the *Br. abortus* ring test antigen has, to a large extent, been nonspecific and that many of the so-called positive ring test reactions have, in

From the College of Veterinary Medicine, Ohio State University, Columbus. Presented in partial fulfillment of the requirements for the degree of master of science, Ohio State University.

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reality, been a combination of a ring test reaction and a Schern-Gorlisch[8] reaction. The Schern-Gorlisch reaction is a test for heat treatment of milk. The test is carried out by adding 1 ml. of milk to 1 drop of a 2 per cent suspension of red blood cells or 1 drop of a 1 per cent suspension of bone charcoal, after which the sample is shaken and incubated at 37 C. for two hours. In the instance of raw milk, the blood cells or bone charcoal apparently adhere to the fat droplets and are carried up to form a red or black ring at the top. The ability of these to adhere during the rising is probably dependent on the amount of fat, as well as the size of the fat droplets, and the quality of the protein layer surrounding the fat droplets. These factors vary from 1 animal to another and, for this reason, the Schern-Gorlisch reaction functions best on composite samples of several animals, as the individual variations are thereby somewhat eliminated.

The ring test was first described by Fleischhauer.[3] As a result of his investigations, Fleischhauer concluded that, in the testing of herd samples, only reactions which occur within twenty minutes following incubation could be considered as positive. The majority of all herd samples would show ring formation forty minutes after incubation. These reactions were considered as being nonspecific or Schern-Gorlisch reactions.

Smitmanns[11] concluded that reactions on herd samples should be considered positive if the reaction started within twenty-five minutes following incubation and if, after two hours' incubation, there were a 2- to 4- mm. wide blue-violet ring sharply defined from the underlying white milk.

Fleischhauer and Herman[4] found that, in the investigation of composite samples, the time of the beginning of the reaction and not the end was important. Reactions which started after thirty minutes no doubt had less agglutinins in the milk and many of these were due to Schern-Gorlisch reactions. These late reactions are borderline cases and are only of such value as they can be further substantiated by other known methods, such as the whey agglutination, rapid agglutination, and fresh milk agglutination.

Norell and Olson,[5] of Sweden, published results of investigations on the value of serologic milk examinations by means of the ring test. The ring test antigen which they used apparently gave the best results so far. They incubated herd samples for fifty minutes, without obtaining a particularly great number of nonspecific reactions. The ring test proved to be far more sensitive on herd samples than did the slow, whey-agglutination test.

In further investigations to evaluate the applicability of the ring test as a diagnostic agent in combating brucellosis,[6] the blood test was more sensitive than the ring test, despite the fact that the agglutination titer for a positive reaction had been placed at complete at 1 : 20. When results obtained from the herd samples of milk were compared with blood tests of individual animals, there was agreement in 94.6 per cent of the cases.[5]

Winther and Hansen[10] compared the ring test and whey test in 548 samples of mixed milk (can milk) and 254 tests of milk from individual cows. The results correspond to previous information in that approximately 5 per cent of the tests gave a positive and 5 per cent a doubtful reaction to the ring test, whereas, the slow agglutination test was negative. In no instance was the ring test negative and the slow agglutination test positive.

When the results with herd milk ring tests were compared with the blood tests of individual animals, Seit and Jorgensen,[9] of Denmark, found only a 3.8 per cent disagreement.

Christiansen[2] reported results on 6,266 herds that had been blood tested at about the time of performing the milk ring test. There was a complete agreement between the two tests in 93.2 per cent of the tested herds, partial disagreement in 2.4 per cent, and complete disagreement in 4.4 per cent, when only one ring test was taken into account. Ring tests carried out simultaneously with blood tests on

TABLE I—Comparison of Results of Blood Tests with Individual Milk Ring Tests

Herds	No. animals	Agreement of ring and blood tests			Disagreement		Efficiency in locating infected animals (%)
		Blood − Ring −	Blood + Ring +	%	Blood + Ring −	Blood − Ring +	
Herd A	63	54	5	93.7	0	4	100.0
Herd B	24	9	3	50.0	0	12	100.0
Herd C	19	5	14	100.0	0	0	100.0
Herd D	31	5	20	80.7	1	5	95.2
Herd E	41	13	8	51.2	0	20	100.0
Herd F	32	12	10	68.7	1	9	90.9
Herd G	10	9	1	100.0	0	0	100.0
Herd H	168	160	2	96.4	0	6	100.0
Herd I	40	12	14	65.0	1	13	93.3
Total	428	279	77	83.2	3	69	96.3

3,952 individual animals showed an 84.3 per cent agreement, partial disagreement in 4.1 per cent, and total disagreement in 11.6 per cent of the instances. In 82.3 per cent of the instances of total disagreement, the ring test was positive and the blood test negative. There was noticeably less agreement between the blood and the ring tests when these tests were applied to samples from cows recently fresh, from strippers, or from cows infected with mastitis.

Bruhn[1] reported that, by means of the ring test, he was able to detect 97 of 121 (80.2%) cows which were blood reactors at 1 : 20 or higher. There were 24 instances of positive blood and negative ring tests. The ring test failed in 3 instances, in 1 with the blood-agglutination titer 1 : 100 and in 2 cases with the titer 1 : 200. Bruhn concluded that these negative ring test reactions in samples from individual cows which are strong reactors are usually due to the lack of cream-rising capacity of the milk. When these samples are mixed with ring test-negative pooled milk, positive reactions are obtained.

Roepke, Clausen, and Walsh,[6] in reporting results of preliminary studies on slightly over 6,000 herds in Minnesota, found a 75 per cent efficiency for the milk and cream ring test when compared to the blood serum-agglutination test in locating infected herds.

Roepke, Paterson, Driver, Clausen, Olson, and Wentworth,[7] in reporting subsequent studies in Minnesota, found an agreement between the ring test and the blood serum-agglutination test on 96.2 per cent of 8,469 herds tested. The ring test was positive for 68 per cent of 385 herds considered infected on the basis of blood tests. Studies in 107 infected herds which were negative to the ring test revealed that, in 69 herds (65%), the reactor animals were not in production at the time of milk collection. The ring test was positive for 88 per cent of the herds in which infected animals were contributing to the can sample.

From the foregoing observations, several facts can be deduced. These suggested the modification used in the following work:

1) The milk ring test is generally considered as being more efficient in detecting Brucella infection when used with composite rather than individual milk samples.

2) Variations in the cream-rising capacity of milk are responsible for many discrepancies in the milk ring test.

3) The milk ring test, as presently used, is applicable only to whole milk possessing normal cream-rising capacity.

Materials and Methods

Blood and milk specimens from nine Brucella-infected herds, comprising 428 animals, were investigated; in eight of these herds, infection had been present for at least two years. At the time of sampling, infection had been present for less than a year in herd H.

Samples of milk were collected in rubber-stoppered glass tubes 20 by 150 mm. and held in the refrigerator until used. Usually, approximately 10 ml. were collected at the regular milking time. Composite quarter samples of either foremilk or strippings were used. The milk samples seldom were held more than eight hours before testing; many were tested within two to three hours after collection.

Blood specimens were taken at the time of collection of the milk samples, refrigerated, and submitted to test the next day.

The stained antigen was prepared according to the technique described by Roepke, Clausen, and Walsh.[6] The stock. *Br. abortus* tube antigen (about 4% cells) was obtained from the U. S. Bureau of Animal Industry. This stock antigen was washed with saline by centrifugation and weighed following decanting to obtain the weight of the bacterial paste. The paste was stirred thoroughly while adding a few drops of saline to obtain a smooth, thick, creamy suspension of cells in a small volume of saline. This suspension was then added to the staining solution.

The staining solution was prepared as follows:

1) Nine grams of ammonium aluminum

TABLE 2—Comparison of Results of Blood Tests with Individual Capillary Tests

Herds	No. animals	Agreement of capillary and blood tests			Disagreement		Efficiency in locating infected animals (%)
		Blood − Cap. −	Blood + Cap. +	%	Blood + Cap. −	Blood − Cap. +	
Herd A	63	54	5	93.7	0	4	100.0
Herd B	24	20	3	95.8	0	1	100.0
Herd C	19	5	13	94.7	1	0	92.9
Herd D	31	19	9	96.5	2	1	90.5
Herd E	41	31	8	95.2	0	2	100.0
Herd F	32	19	10	93.5	1	2	90.9
Herd G	10	9	1	100.0	0	0	100.0
Herd H	168	159	2	96.4	0	7	100.0
Herd I	40	15	14	72.5	1	10	93.3
Total	428	321	75	92.5	5	27	93.8

sulfate were dissolved in 100 ml. of distilled water, and 30 ml. of glycerin were added.

2) One gram of hematoxylin was dissolved in 2 ml. of alcohol (95%) by heating to 50 C. After dissolution, distilled water was added to make a volume of 100 ml.

3) Solutions 1 and 2 were mixed, and to this mixture was added 0.17 Gm. of sodium iodate dissolved in 2 ml. of distilled water.

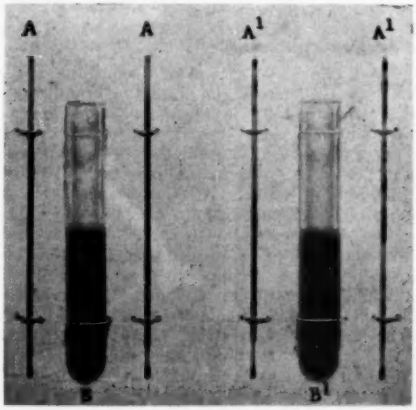

Fig. 2—Milk test reactions, ten minutes from start of test. A, negative milk, capillary tube test; A^1, positive milk, capillary tube test; B, negative milk, ring test; and B^1, positive milk, ring test.

Fifteen minutes were allowed for oxidation to take place. The mixture was diluted 1 : 5 with saturated ammonium aluminum sulfate (approximately 10%). The diluted stain was allowed to stand for twenty hours at room temperature. Insolubles were removed by filtration. The staining was brought about by adding the bacterial suspension to the dye solution, using about 10 Gm. of the paste to about 1,200 ml. of the diluted stain. The suspension was placed in a hot water bath with gentle stirring until the mixture reached 65 C. This temperature was held for three to five minutes, when the material was cooled. The suspension was centrifuged at 2,600 revolutions per minute to collect the cells, decanted, and washed about three times using saline acidified to pH 3.5 to 4.0 with hydrochloric acid.

The stained cells were suspended in a 50-50 mixture of glycerin and saline containing 0.5 per cent phenol. The final bacterial cell concentration was about 4 per cent by volume, determined by the centrifuge method with Fitch-Hopkins vaccine tube.

Capillary tubes used in this test were made of soft glass, approximately 0.8 by 90.0 mm. The test was performed by allowing the stained antigen to be drawn into the capillary tube approximately 5 to 7 mm. After milk samples had been thoroughly mixed, the end of the capillary tube containing antigen was dipped into the milk and the tube was allowed to fill, being sure to avoid entry of air bubbles. The capillary tube was inverted and the end was inserted into the plasticine at an angle of approximately 45 degrees from the horizontal, so as to allow the antigen to migrate slowly through the milk. The tubes were allowed to stand for thirty minutes at room temperature and were read against a white background. The use of a magnifying glass and light, such as is shown in fig. 1, proved to be beneficial.

The milk ring tests were performed according to the techniques described by Roepke.[7] The same antigen, described elsewhere in this paper, was used in conducting both the ring tests and the capillary tests.

The blood serum agglutinin titers were determined by the standard test tube method.

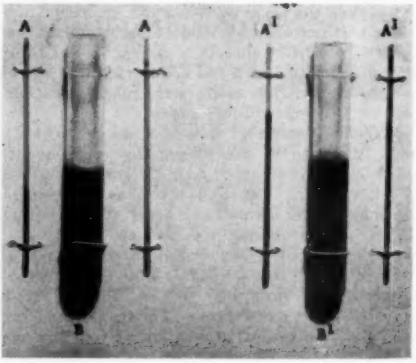

Fig. 3—Milk test reactions, thirty minutes from start of test. A, negative milk, capillary tube test; A^1, positive milk, capillary tube test; B, negative milk, ring test; and B^1, positive milk, ring test.

It was set up to include dilutions of 1 : 25, 1 : 50, 1 : 100, and 1 : 200. Standard *Br. abortus* antigen, furnished by the Bureau of Animal Industry for the testing of cattle in the bovine brucellosis control program, was used in all the tests.

The blood serum-agglutination test was used as the standard in the determination of the efficiency of the ring test and the capillary test in locating infected animals.

RESULTS

The antigen, in Brucella-negative milk,

forms a smooth, fine line along the lower side of the capillary tube. The antigen, in Brucella-positive milk, shows a clumping or agglutination up and down the tube. The tube should be rotated between the forefinger and the thumb at the time of reading to observe the line of antigen on the underside of the tube.

Figure 2 illustrates the capillary tube test and ring test with negative and positive samples in whole milk ten minutes after setting the tests. Even at this time, the reaction is obviously positive in the capillary tube, while no change is visible in the ring test. Figure 3 shows the same milk samples set thirty minutes, and fig. 4, the same samples set for ninety minutes. A comparison of fig. 2, 3, and 4 shows the relative speed and clarity of the reactions of the two tests over a period of ninety minutes.

While whole milk samples were used in all cases as the test material in this report, in a limited number of tests, corresponding results have been obtained using skimmed and homogenized milk samples in place of the whole milk.

Table 1 is a summary of the results obtained in nine herds comprising 428 animals in which infection was known to exist. The milk ring test and the blood serum-agglutination test were in agreement in 83.4 per cent of the instances, while being 96.3 per cent efficient in detecting infected animals.

Table 2 records results obtained when the milk capillary tube test was compared to the blood serum-agglutination test on the same animals reported in table 1. In 92.7 per cent of the cases, the capillary tube test agreed with the blood test, while a 93.8 per cent efficiency in detecting infected animals was noted.

It is apparent from the data presented thus far that the capillary tube test compares quite favorably with the milk ring test, both in agreement with the blood test and in the percentage of efficiency in locating infected animals.

It is interesting to note the apparent reduction in the number of animals which were positive to the ring test but negative to the blood test (false positive ring test) when the capillary test was used. A relatively large number, 68 of 347 (19.6%), of blood-negative animals were found to be positive to the ring test, while only 26 of 347 (7.5%) false positives were found when the capillary test was employed.

These false positive reactions are particularly objectionable from the standpoint of added expense and the confusion which arises when any test of this kind is found to be in error.

This difference was probably due to the fact that one plus and sometimes two plus

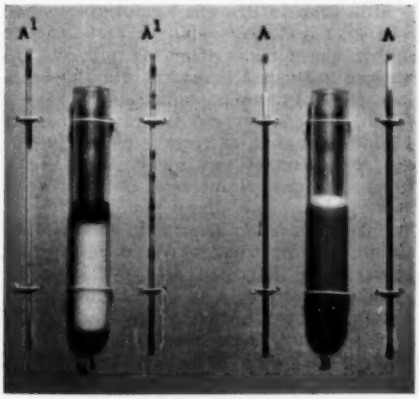

Fig. 4—Milk test reactions, ninety minutes from start of test. A, negative milk, capillary tube test; A^1, positive milk, capillary tube test; B, negative milk, ring test; and B^1, positive milk, ring test.

ring test reactions usually give negative capillary readings, thus eliminating a large number of doubtful readings.

Of particular significance are the saving in the amount of antigen used and the time consumed in conducting the capillary tube test. Approximately, 60 ml. of stained antigen were used in conducting the milk ring test on the 428 samples, while less than 5 ml. were needed when the capillary tube test was used on the same samples.

Less than one-half the time is consumed in conducting the capillary tube test, as compared to the time required in performing the milk ring test.

DISCUSSION

A comparison of the results of the capillary tube test with those of the milk ring test in the present study indicates that there is good agreement between the two. On the basis of these results, it seems reasonable to assume that the capillary tube test is more effective than the ring test in diagnosing Brucella infection in individual animals, and may be substituted for the

ring test in detecting infection in herd samples.

Approximately three times as many false positive reactions occurred when the milk ring test was used. The capillary tube test seemed, therefore, to be somewhat superior in the over-all agreement with the blood test.

Milk high in Brucella agglutinin content will give a strong capillary test reaction within five minutes after the milk and the stained antigen are drawn into the capillary tube. Individual milk samples low in agglutinin content require more time for visible agglutination to take place. During the early part of this investigation, readings were made at intervals of ten, fifteen, thirty, sixty, and ninety minutes at room temperature, in an attempt to establish optimum reading time for the capillary test. It was found that approximately 95 per cent of all positive reactions were visible within fifteen minutes following the start of the test and that there was little change in readings following the thirty-minute period. The capillary test results reported in this paper were based on a thirty-minute reading time, when set at room temperature.

Whole milk was used as test material in all cases reported in this investigation. However, milk containing little or no cream and milk in which the cream-rising capacity has been altered may also be tested by the capillary tube method.

If properly conducted, the capillary tube test is apparently reliable and may have several applications and possible advantages over the milk ring test, *viz*:

1) It can be used to detect Brucella agglutinins in skimmed and homogenized milk as well as whole milk.

2) Only about 5 per cent as much stained antigen is required in conducting the capillary tube test.

3) Less than one-half as much time is consumed in conducting the capillary tube test.

4) Capillary tubes used in performing the test are inexpensive and may be discarded following use, thus reducing considerably the expense of cleaning equipment.

The experimental data presented in the foregoing should be considered as preliminary in nature. First, the number of animals used was small. Second, the blood serum-agglutination test which was used as a standard in measuring the efficiency of these milk tests possesses certain limitations, at best.

SUMMARY

1) A simple, practical, and economical milk test is described for the detection of Brucella-infected animals. Skimmed, homogenized, and whole milk may be used as test material.

2) A comparison was made of the ring test, the capillary tube test, and the blood serum-agglutination test on 428 animals. The ring test agreed with the blood test in 83.4 per cent of the instances, while detecting 96.3 per cent of the animals positive to the blood-agglutination test. The capillary tube test found 93.8 per cent of the infected animals, while agreeing with the blood results in 92.7 per cent of the cases.

3) The observations made so far suggest that the capillary tube test can be used to detect Brucella infection in individual animals.

4) The data obtained in this preliminary study seem sufficiently significant to justify more comprehensive investigations to determine the optimum concentration of antigen and the value of the test in indicating infection of the animals in the interval between blood tests.

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Experimental Evaluation of Six Halogenated Sulfapyrimidines in Cattle

E. C. McMANUS, B.S., D.V.M., Ph.D.; S. F. SCHEIDY, V.M.D.; B. J. ANGSTADT, B.S.; S. E. McKINNEY; A. A. PITT, B.A.; G. S. SCHUCHARDT, B.S.

Glenolden, Pa.

THE INTRODUCTION of the pyrimidine ring into the sulfonamide structure has brought about the most outstanding improvement in this type of compound. Three pyrimidine sulfonamides (sulfadiazine, sulfamerazine, and sulfamethazine) are used extensively by the medical professions. The drugs are characterized by a high degree of antibacterial activity, a low order of toxicity, good absorption from the intestinal tract, and a slow excretion rate.

In the course of continued investigations of sulfapyrimidine derivatives by Beyer,[1] it was found that halogenation of the number five carbon of the pyrimidine ring in some cases lowered the renal clearances of the compounds in dogs. The advantage of the low renal clearance is apparent, and it was decided that the compounds merited study in other species. The purpose of this report is to describe a pharmacologic study of halogenated sulfapyrimidines in cattle.

In all, nine sulfapyrimidines were compared. They included sulfadiazine, sulfamerazine, sulfamethazine, and the six corresponding compounds wherein either a chlorine or a bromine atom was attached to position five of the pyrimidine ring. The structures are illustrated in fig. 1.

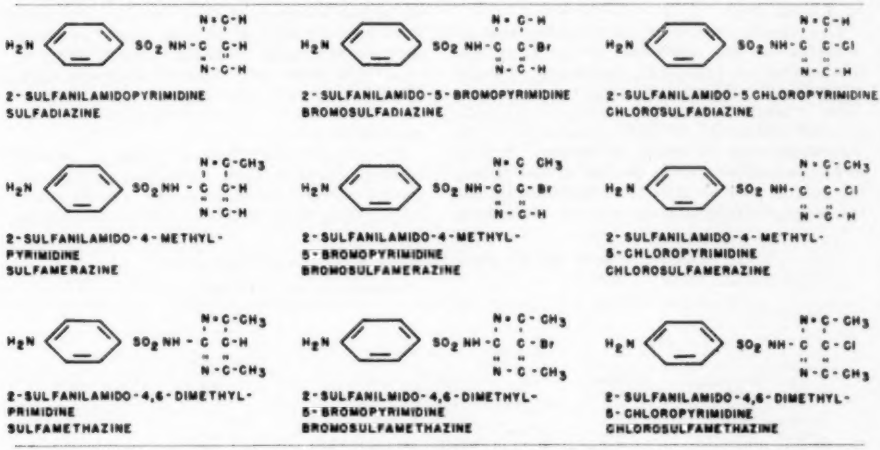

Fig. 1—Structures of nine sulfapyrimidines.

EXPERIMENTAL

Normal, healthy calves, ranging in weight from 200 to 450 lb., were used for the plasma concentration and binding studies. The animals were maintained in a comfortable stable during the experiments. Whole oats and mixed hay were fed *ad libitum*. Clean drinking water was available at all times.

Plasma Concentrations Following Oral and Intravenous Administration.—The plasma concentrations were determined following the single administration of oral and intravenous doses of the compounds to the calves. The doses used in all cases were 0.072 Gm. per kilogram of body weight (½ gr./lb.) for intra-

From the Medical Research Division, Sharp and Dohme, Inc., Glenolden, Pa.

The authors express appreciation to Dr. K. H. Beyer for helpful suggestions.

venous injections and 0.14 Gm./kg. of body weight (1 gr./lb.) for oral administrations. Aqueous solutions of the sodium salt were administered intravenously. Tragacanth suspensions of the compounds were given orally. At the intervals shown in the tables, blood samples were drawn for sulfonamide analysis. The conventional Bratton-Marshall[3] colorimetric method was used to determine the plasma concentrations of the compounds. Results are presented in tables 1 and 2.

From the figures presented in tables 1 and 2, it is possible to correlate structural changes and disposition of the drugs. Since the compounds compare somewhat differently when given orally and intravenously, the results by the two routes of administration will be considered separately.

The intravenous data are presented in table 1. In comparing the figures at twenty-four and forty-eight hours after administration for sulfadiazine and sulfamethazine with those for the respective halogenated structures, it is evident that halogenation did not significantly influence the rate of elimination. However, in the case of sulfamerazine, halogenation caused a decided change. Bromo- and chlorosulfamerazine maintained plasma concentrations substantially greater than did sulfamerazine itself, the two compounds being approximately equal to sulfamethazine with respect to persistence of plasma level.

The plasma concentrations following oral administration are influenced by an additional factor, that of intestinal absorption. Results of these experiments are presented in table 2. Here, a more marked superiority of the halogenated analogues of sulfamerazine over the nonhalogenated structure is shown. Bromo- and chlorosulfamerazine persist in the plasma for greater periods than do sulfamethazine and the other sulfapyrimidines studied. It appears that halogenated sulfamethazine also surpasses sulfamethazine in these same oral experiments but, unfortunately, only total values were obtained for this compound and, from the data, a direct comparison with sulfamethazine can not be made. The fact that the halogenated sulfamerazines (and probably the halogenated sulfamethazines) attain and maintain higher plasma concentrations than do the remainder of the sulfapyrimidines when administered orally, while at the same time they are only approximately equal in persistence to sulfamethazine by the intravenous route, suggests that halogenation increases the degree of intestinal absorption.

It is evident that acetylation of bromosulfamerazine and of chlorosulfamerazine is appreciable. The amount of acetylation in the case of bromosulfamerazine appears to be greater than in the case of bromosulfamethazine. Unfortunately, acetylated and nonacetylated values are not complete for the nine compounds, so that a full comparison with respect to acetylation is not possible.

Acute Toxicity.—Acute toxicity experiments were performed in mice. Carworth CF-1 female white mice weighing between 16 and 20 Gm. were given injections at a constant rate of 0.5 cc. per minute. Administration was made into the tail vein. The dosage was calculated in milligrams of the acid form of the compound per kilogram of body weight. Ten mice were used for each of at least four dose levels covering the range from $l.d._0$ (lethal dose) to $l.d._{100}$ The mice were observed for seven days. The $l.d._{50}$ was calculated according to the method of Miller and Tainter.[3] Results are summarized in table 3.

Plasma Protein Binding.—Plasma protein binding determinations were done using calf plasma samples taken at a high and at a low sulfonamide concentration. The ultrafiltration

TABLE I—Plasma Concentrations Following Intravenous Administration

Compound		Hours after administration						
		1	4	8	12	24	36	48
0.072 Gm./kg. (½ gr./lb.)		mg. per 100 cc.						
Sulfadiazine	Total	...	...	...	...	..	..	..
(Ave. 6 Animals)	Free	14.6	8.5	5.2	...	1.3	..	<1.0
Sulfamerazine	Total	...	...	...	...	..	..	..
(Ave. 6 Animals)	Free	15.3•••	13.6	7.8	...	2.5	..	<1.0
Sulfamethazine	Total	...	...	...	...	..	..	..
(Ave. 6 Animals)	Free	17.0••	14.8••	10.9	...	5.1	..	1.5
Bromosulfadiazine	Total*	21.3	12.5	9.6	5.2	2.0	1.2	<1.0
(Ave. 3 Animals)	Free	...	...	...	...	..	..	..
Bromosulfamerazine	Total	26.3	21.8	19.1	15.3	7.3	3.3	2.0
(Ave. 3 Animals)	Free	21.6	17.6	17.5	14.5	4.9	2.2	1.2
Bromosulfamethazine	Total	27.0	22.6	19.6	14.7	5.9	2.0	1.4
(Ave. 3 Animals)	Free	22.7	21.5	18.1	14.0	5.3	1.7	<1.0
Chlorosulfadiazine	Total	20.3	12.2	8.7	4.9	1.8	<1.0	<1.0
(Ave. 3 Animals)	Free	...	...	...	...	..	..	..
Chlorosulfamerazine	Total	23.1	17.1	13.6	10.4	5.5	2.7	1.7
(Ave. 3 Animals)	Free	20.3	14.5	11.1	8.0	3.8	1.7	1.0
Chlorosulfamethazine	Total	21.0	17.6	14.0	12.2	4.9	<1.0	<1.0
(Ave. 3 Animals)	Free	...	...	...	...	..	..	..

*Total dialyzable sulfonamide after acid hydrolysis of the plasma. This is considered to measure unaltered plus acetylated compound. **Average of 5 animals. ***Average of 4 animals.

method described by Lavietes[4] was used. The results are summarized in table 4. Each value represents a determination from the heparinized plasma of 1 animal.

TABLE 2—Plasma Concentrations Following Oral Administration

Compound		Hours after administration								
		1	2	4	8	12	24	36	48	72
0.14 Gm./kg. (1 gr./lb.)		mg. per 100 cc.								
Sulfadiazine	Total	..	..	...	...	...	...	...	...	..
(Ave. 6 Animals)	Free	0.8	1.6	3.0	4.2	...	4.3	...	2.9	..
Sulfamerazine	Total	...	...	...	...	...	...	...	...	..
(Ave. 6 Animals)	Free	1.5	2.2	4.9	7.5	...	6.4	...	2.7	..
Sulfamethazine	Total	..	..	...	...	...	...	...	...	..
(Ave. 6 Animals)	Free	3.4	6.7	9.5	11.1	...	9.3	...	3.9	..
Bromosulfamerazine	Total	3.4	6.5	8.5	18.1	19.0	16.4	14.3	8.4	5.2
(Ave. 3 Animals)	Free	2.9	5.6	6.4	14.0	17.0	11.6	9.3	5.0	2.8
Chlorosulfameraxine	Total	5.0	9.0	13.4	16.8	18.5	18.4	15.1	10.0	4.1
(Ave. 3 Animals)	Free	4.6	8.0	11.6	14.1	15.3	14.6	10.7	6.8	2.6
Chlorosulfamethazine	Total	2.1	3.9	7.9	12.1	15.0	16.1	11.8	7.2	1.8
(Ave. 3 Animals)	Free	..	..	...	...	...	...	...	...	..

It is clear, from the results presented, that halogenation increases the degree of plasma protein binding. How this property of the drugs would affect their therapeutic efficacy is not known with certainty. It is well known, from the work of Davis,[5] that plasma protein binding influences distribution of sulfonamides into extravascular fluids. Also, he has presented evidence to show that only the unbound fraction of the sulfonamide is available for antibacterial action. However, therapeutic studies that indicate that sulfadiazine, sulfamerazine, and sulfamethazine with equal blood concentrations are comparable in activity[6] have been reported; yet there is a significant difference in degree of plasma protein binding of the three compounds.

TABLE 3—Mouse Intravenous Acute Toxicity

	i.v. acute $l.d._{50}$ (mg./kg.)	Standard error $P = 0.05$
Sulfadiazine (10 Animals)	800	± 79
Sulfamerazine (10 Animals)	1,200	±104
Sulfamethazine*	....	....
Bromosulfadiazine (10 Animals)	280	± 58
Bromosulfamerazine (10 Animals)	660	± 53
Bromosulfamethazine (10 Animals)	490	± 41
Chlorosulfadiazine (10 Animals)	230	± 86
Chlorosulfamerazine (10 Animals)	710	± 42
Chlorosulfamethazine (10 Animals)	500	± 45

*Mouse intravenous (i.v.) acute toxicity of sulfamethazine has not been determined in these laboratories. Rose, Martin, and Bevan[7] reported the i.v. acute $l.d._{50}$ to be approximately 700 mg. per kilogram of body weight. Schmidt *et al.*[8] reported the mouse intraperitoneal acute $l.d._{50}$ to be approximately 900 mg. per kilogram of body weight.

SUMMARY

Sulfadiazine, sulfamerazine, and sulfamethazine were compared in cattle with the six corresponding halogenated sulfapyrimidines wherein either a chlorine or a bromine atom was attached to position five of the pyrimidine ring.

In respect to persistence in the plasma, halogenation accomplished the greatest change in the case of sulfamerazine. Bromo- and chlorosulfamerazine, administered intravenously, maintained plasma cencentra-

TABLE 4—Plasma Protein Binding

	Total (mg./100 cc.)	Unbound (mg./100 cc.)	Bound (mg./100 cc.	Bound (%)
Sulfadiazine	13.2	9.4	3.8	29.0
(1 Animal)	2.7	1.8	0.9	33.0
Sulfamerazine	14.7	8.6	6.1	42.0
(1 Animal)	2.2	1.1	1.1	50.0
Sulfamethazine	16.7	6.6	10.1	61.0
(1 Animal)	3.4	1.0	2.4	71.0
Bromosulfadiazine	23.0	9.6	13.4	58.0
(1 Animal)	1.4	<1.0	1.4	app. 99.0
Bromosulfamerazine	22.9	3.1	19.8	87.0
(1 Animal)	8.1	1.2	6.9	85.0
Bromosulfamethazine	23.5	4.6	19.0	81.0
(1 Animal)	5.9	<1.0	5.4	91.0
Chlorosulfadiazine	18.6	8.0	10.5	51.0
(1 Animal)	1.7	<1.0	1.7	app. 99.0
Chlorosulfamerazine	26.7	5.7	20.9	78.0
(1 Animal)	12.3	2.0	10.3	83.0
Chlorosulfamethazine	23.4	3.8	19.6	84.0
(1 Animal)	12.3	1.3	11.0	89.0

tions substantially greater than did sulfamerazine itself, being approximately equal in this respect to sulfamethazine. When administered orally, a more marked superiority of halogenated sulfamerazines and also probably of halogenated sulfamethazines over the corresponding nonhalogenated structures was found. It is thought that the more definite superiority demonstrated when the compounds were given orally is due to more complete intestinal absorption of the halogenated compounds than of the nonhalogenated ones.

Acute intravenous toxicity data were obtained in mice. The figures for the least toxic halogenated sulfapyrimidines, bromo- and chlorosulfamerazine, are close to that for sulfadiazine.

Halogenation of sulfapyrimidines increases the degree of plasma protein binding.

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Nasal Granuloma—A Report of Two Cases in Cattle

VIRGIL B. ROBINSON, D.V.M., M.S.

College Station, Texas

NASAL GRANULOMA is a chronic proliferative disease of the anterior nasal passages of cattle and horses. Cattle are affected most frequently, but several cases have been reported in horses. In the United States, the disease has been reported in cattle only. It is prevalent in India but is relatively rare in other parts of the world, including the United States. The water buffalo of India has not proved susceptible, even when kept in close contact with infected cattle.

Little information is available on the epizoötiology of nasal granuloma or the nature of the etiologic agents. In 1912, Zschokke[13] reported a similar disease in a horse in South Africa, caused by an organism which he called *Rhinosporidium equi*. In the same country, the disease has been reported in mules[9] and in cattle.[12] *Rhinosporidium seeberi* causes a disease in man not unlike the nasal granulomatosis seen in animals. The majority of reported cases of rhinosporidiosis in man has occurred in Argentina and India, but a few cases have been recognized in other parts of the world.[1] Krishnamurti[7] reported nasal granuloma in a horse and in cattle in India caused by a Rhinosporidium. Rhinosporidium organisms have not been grown successfully on artificial mediums or transmitted to experimental animals. Datta[3] presented experimental evidence to indicate that the etiologic agent in some of his cases was probably the blood fluke, *Schistosoma spindale*. Malkani[8] and Rao[10] confirmed the findings of Datta, and Rao was successful in experimentally reproducing this form of the disease in cattle. It, therefore, appears likely that two types of the disease exist in India.

In 1933, Creech and Miller[2] reported nasal granuloma in a herd of Jersey cattle at the Iberia Livestock Experiment Station, Jeanerette, La. This was the first time the disease was reported in the United States. In the four-year period between 1928 and 1932, 13 animals out of a herd of 60 showed definite clinical symptoms of nasal granuloma. The age range of affected animals included yearling heifers and mature cows. No infected bulls were reported. Some of the animals contracted the disease while in contact with barns, while others contracted it after several months on pasture. Difficult nasal respiration and a mucopurulent discharge were the basic clinical manifestations described. The intensity of the symptoms seemed to increase in the summer and decrease in the winter. The lining membranes of the anterior nasal passages were thickened and heavily studded with nodules up to 2 mm. in diameter. Histologic examination revealed granulation tissue, an infiltration of round cells, and masses of eosinophils. No well-formed giant cells were observed. A limited number of small round bodies were seen in some sections from acute lesions. These were described as having a fairly definite outline and, in some, the border appeared serrated. These bodies were not associated with the disease by these workers. All efforts to grow the causative organisms on artificial mediums and to transmit the disease to experimental animals were unsuccessful. They concluded that the disease in Louisiana was parasitic in nature and probably caused by Schistosoma organisms.

The conclusions of Creech and Miller caused Dikmans[5] to investigate the Louisiana outbreak from a parasitic point of view. After a thorough study, he concluded that this outbreak was not caused by Schistosoma. He associated the small round bodies found in the lesions and nasal secretions with the cause of the condition and suggested that they might be closely related to the genus Rhinosporidium.

In 1936, Davis and Shorten[4] reported a similar case in a cow that was presented for slaughter at a Denver, Colo., meat-packing plant. The chief symptoms were difficult nasal breathing, a copious nasal discharge, and "snoring" breathing sounds. The gross pathology was limited to a nodular thickening of the lining membranes of the anterior nasal passages. On histologic examination, they found a granulomatous structure infiltrated with masses of eosinophils and numerous giant cells. Moderate numbers of lymphocytes, neutrophils, and plasma cells were also present. Many small, encapsulated bodies with granular cytoplasm were observed both in the giant cells and in the tissue spaces. Many small, round eosinophilic bodies were observed near the base of the glandular epithelium. They felt that these bodies were probably immature forms of the causative organisms but were unable to isolate a definite etiologic agent.

From the Department of Veterinary Pathology, Texas A. & M. College, College Station. At present, Dr. Robinson is on leave for graduate study at Vanderbilt University, Nashville, Tenn.

The author wishes to express appreciation to Dr. Hilton A. Smith, head, Department of Veterinary Pathology, Texas A. & M. College, for making available the material for case 2, and for valuable consultation; and to Mr. Geo. N. Griffin for preparing the photographs.

CASE 1

The subject of this report was a 9-year-old family milk cow of mixed breeding. The case originated at Sommerville, Texas, in 1948. This was the only cow on the premises, and she had been raised from a calf by the owner. Respiratory noises following extreme exercise and a nasal discharge had been observed for six months. The symptoms grew worse and she was presented at the large animal clinic of the School of Veterinary Medicine, Texas A. & M. College, College Station. Nasal respiration was extremely difficult. A copious amount of clear exudate, streaked with puslike strands, persisted at both anterior nares. Nasal breathing sounds could be heard several feet away while the animal was at complete rest. She stood with the nose extended, and the increased use of the intercostal and abdominal muscles for respiration was evident. A ballooning of the cheeks occurred when the mouth was closed during expiration and, at times, she resorted to oral breathing. There was no deformity of the external structures about the nose. The animal took food sparingly and, consequently, was in poor physical condition. Masses of nodular growths were present bilaterally just inside the anterior nasal openings. A biopsy was secured for microscopic study. A fungous infection was suspected, but a definite diagnosis was not made from the frozen sections. The condition of the animal continued to deteriorate, and the owner agreed to euthanasia. A postmortem examination was performed immediately after death.

Postmortem Findings.—Macroscopic Observations.—There was an over-all thickening of the membranes lining the anterior nasal passages by what appeared to be dense granulation tissue. Superimposed on the surface was an almost continuous mass of soft nodular growths from ½ to more than 2 cm. in diameter (fig. 1). The mucosa was thickened to the extent that the passages were greatly reduced in size and, in some localities, the lumen was completely obliterated. There was a scattering of petechial and ecchymotic hemorrhages visible near the surface. Many localized areas appeared acutely congested. Numerous yellowish white pinpoint foci were visible from the surface of the nodules but, in most instances, epithelium seemed to cover them. The lesions were bilateral in about the same degree. No appreciable distortion of the nasal septum or the turbinate bones had occurred (fig. 2). The lesions were restricted entirely to the first 6 in. of the anterior nasal passages. No other lesions pertinent to this report were observed.

Histopathology.—The tissue was fixed in 10 per cent formalin and stained with hematoxylin and eosin. A study of the sections gave the impression of chronic proliferative inflammation. In some areas, the epithelium was intact in all its strata except for focal areas of apparent erosion around aggregates of reactive cells near the lower margin. Over most of the surface, the epithelium was in various states of deterioration, even to the point of complete sloughing. Much of the epithelium had a ragged and unhealthy appearance. There was hyperplasia of both the glandular and squamous types. Hyperactivity of the glands was suggested by the numerous vacuoles in the glandular epithelium. Eosinophils had invaded the tissue spaces in large numbers and were seen invading even the more normal layers of stratified squamous epithelium and the glands. It was likely that large masses of eosinophils made up the white pinpoint foci seen on macroscopic examination. A moderate number of neutrophils were present and they were congregated in greatest numbers where the epithelium was denuded. Round cells and well-formed Langhans'-type giant cells were present throughout the sections. There was considerable proliferation of capillaries and connective tissue. The fibroblasts and fibers were loosely arranged so as to leave generous tissue spaces. There were many small round bodies of various sizes in all the paraffin sections studied. All of these had a well-formed cell wall which, in many instances, appeared uneven in thickness. The majority had a blue-staining nucleus which seemed to be made up of several small, sporelike structures. In this form, the nucleus was surrounded by faintly stained, pink cytoplasm and a rather dense cell wall. One of this type had a budlike projection not unlike reproduction in certain types of fungi (fig. 3). A few of the organisms seemed to be made up entirely of small, light pink granules and a cell wall. Many of the bodies were in giant cells, while others seemed to be free

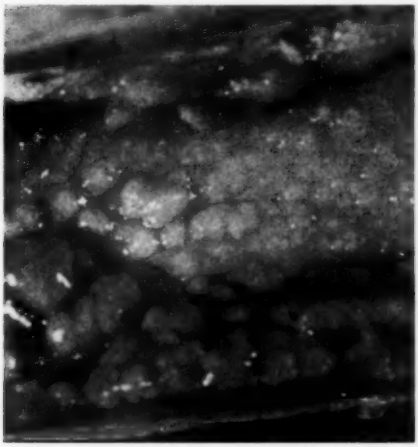

Fig. 1—A close view of the lining of the anterior right nasal passage of case 1. Small white foci are present on the surface of the nodules.

Fig. 2—A dorsal view of the anterior nasal passages and septum of case 1. The passages are seriously obstructed, but the bony structures are not deformed.

Fig. 3—Two fungus-like structures in a giant cell from case 1. One has a budlike projection which may be a form of reproduction.

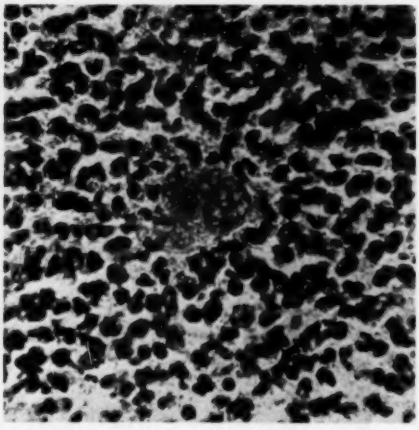

Fig. 4—A clump of three fungus-like structures, free in the tissue spaces but surrounded by an inflammatory type of reaction. Most of the reactive cells are eosinophils, but some mononuclear forms are present. This lesion is from case 1.

in tissue spaces. In many instances, a pronounced inflammatory cell response had occurred around the structures when they were in the tissue spaces (fig. 4). These localized reactions around the organisms left little doubt that these structures were the cause of the lesions. Numerous aggregates of round or oval eosinophilic bodies were present in the epithelium near the basement membrane (fig. 5). These bodies varied in size but averaged about one-half the diameter of erythrocytes. The aggregates were usually surrounded by somewhat of a retaining wall. A few were seen scattered in the tissue spaces and in the lumens of the glandular acini. The bodies were not definitely identified, but it appears likely that they represented a developmental stage of the causative organism. They were probably the same eosinophilic bodies described by Davis and Shorten.[4]

CASE 2

The subject of this report was a young cow whose exact age was not known. She was purchased at a livestock auction by a dealer who was the owner at the time of observation. The case seemed to have originated in the vicinity of Bryan, Texas, in the autumn of 1949. Soon after purchase, the owner noticed a difficulty in respiration and a nasal discharge. She was presented at the large animal clinic of the School of Veterinary Medicine, Texas A. & M. College.

A severe, bilateral, nasal, respiratory impairment was conspicuous. The anterior nares were almost completely occluded by new nodular growths. One of these nodules was removed surgically and referred to Dr. Hilton A. Smith for microscopic diagnosis. Lesions on frozen sections were not conclusive, but a diagnosis of nasal granuloma was made from the paraffin sections. An entirely unfavorable prognosis was advanced and, at the owner's request, the case was dismissed from the clinic. The animal was marketed for slaughter and a postmortem examination was not possible.

Histopathology. — The epithelium was usually intact but in a few localized areas it had sloughed away. Extremely large numbers of eosinophils had invaded the tissue spaces and the epithelial layers. Neutrophils, round cells, and Langhans'-type giant cells were present in moderate numbers. There was glandular and squamous epithelial hyperplasia and hyperactivity of the glands. In some instances,

Fig. 5—Eosinophilic bodies in the stratified nasal epithelium of case 1. The bodies vary considerably in size within the individual clumps.

the glands had become cystic, probably from an infolding of the mucosa and pressure on the emptying ducts. Proliferation of the capillaries and connective tissue had occurred and a loose stroma had been laid down. The capillary endothelial cells were large and the capillaries unusually conspicuous. Several of the fungus-like bodies described in case 1 were seen in each section. None of the small eosinophilic bodies were observed. Otherwise, the pathologic changes did not differ appreciably from those of the preceding case, except that they may have been of shorter duration. No attempt was made to culture the causative organisms or to transmit the disease to experimental animals in either case.

SUMMARY

Two cases of nasal granuloma in cattle which originated in east central Texas are reported. These cases are apparently the third and fourth reported incidents of this disease in the United States. The condition reported here is no doubt the same as that described by Creech and Miller[2] and by Davis and Shorten.[4] The tissue reactions indicate that the bodies seen in the lesions are the cause of the disease. The limited evidence available here also indicates the causative organism is probably a fungus. The morphology does not appear incompatible with that of the genus Rhinosporidium, although it does not seem identical to *Rhinosporidium seeberi*. There is nothing to indicate these 2 cases are caused by Schistosoma organisms.

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Direct Measurement of Arterial and Venous Pressures in Cattle Using the Electromanometer

A. F. SELLERS, V.M.D., M.Sc., Ph.D., and A. HEMINGWAY, Ph.D.

St. Paul, Minnesota

THE MEASUREMENT of the blood pressure of cattle is a measurement which has not been made frequently either in clinical practice or for basic research purposes. The apparatus previously required for accurate blood pressure determination has been too fragile when used on large animals and too complicated in operation, requiring, for example, expensive and time-consuming photographic recording.[4] Recently, there has been developed a new type of blood pressure recorder which is, in comparison with the older methods, simpler in operation and more convenient for use in a large animal clinic or research unit. Investigators have described this new method of blood pressure measurement for use on human subjects and patients.[8, 9, 10] We have used successfully the instrument described by Rappaport and Sarnoff* to measure blood pressure in 105 normal and abnormal dairy cattle. Adult cows and heifers have comprised the majority. The use of this instrument, with certain modifications required for bovine blood pressure measurements, will be described.

METHOD

A 16-gauge, 3-in. needle is connected by tubing with a fluid reservoir (to which pressure can be applied) and a tambour having a metallic membrane. This system is filled with heparinized sodium chloride solution (1 mg. of heparin in 100 ml. of the sodium chloride solution), and the needle inserted by arterial puncture into the common carotid artery of the animal, in a position as shown in fig. 1.

Pressure fluctuations are transmitted through the fluid column of the needle and tubing to the thin metallic diaphragm which is displaced in the manner of a tambour. The metallic diaphragm forms one plate of an electrical air condenser. As the diaphragm moves in response to pressure changes, the capacity of the condenser varies. The variable condenser forms one arm of a Wheatstone bridge. The change in capacity is registered by the amount of off-balance current in the bridge. A schematic diagram of the electrical circuit is shown in fig. 2. An oscillator generates a high frequency current which is applied through a transformer to an alternating current Wheatstone bridge. Changes in the capacity of the condenser caused by blood pressure changes produce a current change in the electrical leads from the bridge. This off-balance current is amplified and rectified by suitable electronic circuits, and the direct current output of the rectifier is applied to a hot wire directing-writing galvanometer.**

An advantage of this apparatus is that photographic recording is obviated and the record is visible as it is made. Also, a definite advantage is the portability of the machine and the ease of assembling the unit. A disadvantage is the long warm-up period of ninety minutes required for obtaining thermal equilibrium and recording stability after turning on the electric power switch.

Comparison of Plastic and Lead Tubing.—Plastic (Tygon†) tubing of the same internal diameter and wall thickness as the lead tubing supplied with the instrument (⅛-in. bore, 3/64-in. wall) was compared with the latter. This procedure was thought desirable in view of the probable greater convenience of plastic tubing when used on pressure measurements on cattle. A three-way stopcock was used to connect to the instrument (transducer), successively, a short lead tube (9¼ in. long) and a length of plastic tubing suitable for use with cattle (6½ ft. long). The carotid artery of a normal nembutalized dog was cannulated with a 16-gauge, 3-in. needle, with the tip toward the heart. Records were then taken from this cannula, using the plastic and lead tubing, alternately.

A length of lead tubing equal to that of the plastic tubing (6½ ft.) was then prepared, and, using the three-way stopcock as described, records taken from the carotid artery of a normal cow by the method outlined below. Cannulation was with a 16-gauge, 3-in. needle.

From the Division of Veterinary Medicine, University of Minnesota, and the Minnesota Agricultural Experiment Station, St. Paul.

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*This instrument is available commercially as the Electromanometer, manufactured by the Sanborn Company, Cambridge, Mass.

**Viso-Cardiette, Sanborn Company, Cambridge, Mass.

†U. S. Stoneware, Akron, Ohio.

Effect of Bevel Position on Carotid Pulse Records.—For exact measurement of the potential energy (pressure) of blood flowing rapidly (in large arteries), lateral pressure readings alone are desired.[7] Since bevelled needles were being used, the effect of facing the needle bevel toward the heart (upstream) or away from the heart (downstream) was studied in a series of 24 normal dairy cows. The recordings were taken consecutively from the same cannula, on a given animal. A notch on the needle hub indicated the face of the bevel.

Arterial Pressure Recording.—A thirty-minute warm-up period was allowed for the instrument. An area approximately 8 cm. square was clipped and shaved over the middle third of the jugular furrow. Twenty milliliters of 2 per cent procaine hydrochloride solution were used intradermally and subcutaneously, the bulk of the dose being deposited deeply in the vicinity of the common carotid artery. An 18-gauge, 2-in. needle was used to go above and behind the jugular vein to deposit the procaine.

The aspirator bottle and transducer were set at a common level with the needle tip (point of puncture). Preliminary trials demonstrated no necessity for incision of the skin. Sixteen-gauge, 3-in. needles were treated with a silicone preparation‡ inside and out. Needles so treated were sterilized and kept in individual tubes. These siliconed needles were used to cannulate the carotid artery.

Cannulation was performed by thrusting the needle through the skin just below the ventral border of the jugular vein into the common carotid artery, at right angles to the long axis of the vessel. The needle hub was then connected to the plastic tubing fitted with an adapter tip, taking care to exclude air bubbles.

During the above procedure, the head and neck were restrained to one side with a rope halter. Subsequent to cannulation, the animal was allowed complete freedom, other than being stanchioned. After waiting a few minutes to assure lack of apprehension on the part of the animal, recording was started and continued for several minutes. Lack of change of mean pressure with time was taken as a criterion of absence of apprehensiveness or restlessness on the part of the animal, and the final portions of such tracings taken as representative of the normal arterial pulse.

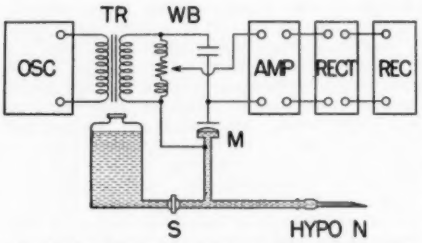
The 100-mm. mercury range of the instrument was employed for the arterial pulse tracings, setting 150-mm. mercury pressure on the center line of the paper.

Venous Pressure Recording.—A ninety-minute warm-up period was allowed for the instrument. Twelve-gauge, 2-in. siliconed needles were used to cannulate the middle third of

‡General Electric Co., Dri-Film No. 9987.

Fig. 1—Electromanometer in use in pressure recording from common carotid artery of dairy cow. Local anesthesia produced by 2 per cent procaine hydrochloride. Apparatus rests on four-wheeled table.

the external jugular vein, after local anesthesia as previously described. After cannulation, the animal was allowed complete freedom as before, and several minutes allowed to elapse before recording. Artifacts (eructations, swallowing movements, coughing, somatic movements) were indicated on the tracing with the



OSC = high frequency oscillator; TR = transformer; WB = Wheatstone bridge; AMP = amplifier; M = metallic membrane; RES = reservoir; RECT = rectifier; REC = recording galvanometer; HYPO N = hypodermic needle; S = stopcock.

Fig. 2—Schematic diagram of electrical connections of manometer.

code marker. Such artifacts were similarly indicated on the arterial pressure tracings, but were of lesser magnitude than in the case of the venous pulse records, as would be expected. The code marker was used to record respiratory movements on both arterial and venous pulse records. This respiratory record was, of course, made subjectively; further, the code marker tip was not able to be aligned vertically with the writing stylus. However, the record was routinely included because of its value in indicating respiratory rate and rhythm. Multichannel recorders would, of course, permit simultaneous observations on respiratory movements, arterial pressure, venous pressure, and electrocardiogram.

The 10-mm. mercury range of the instrument was most commonly employed for the venous pulse tracings; an additional record using the 20-mm. range was included when wave amplitudes were large in the 10-mm. range.

RESULTS

Comparison of Plastic and Lead Tubing.—It will be noted in fig. 3 and 4 that the two types of tubing gave tracings topographically different. This appeared to be true both in the dog, where a section of lead tubing of minimum length was employed for comparison, and in the cow, where lead and plastic tubings were of equal length. Differences were due principally to the presence, in records taken with the lead tubing, of small waves superimposed on a basic pulse contour common to both lead-tubing and plastic-tubing records. These presumably are natural vibrations of the periodic arterial system and reflected waves, not obliterated by damping in the case of the lead tubing.

Since the pulse pressures and general pulse contour observed were quite similar in both cases, it was felt that the more convenient plastic tubing could be used satisfactorily for routine pressure recording.

Effect of Bevel Position on Carotid Pulse Records.—Figure 5 includes tracings illustrating the types of variation encountered. In the series of 24 normal animals in which comparisons were made, 14 animals (58%) showed the topographic change seen in the top pair of tracings of fig. 5, from animal 25. This consisted of a change in the position of a superimposed wave on the ascending limb. This wave was presumably a free or natural vibration, as indicated later. It will be noted that, with the bevel toward the heart, the superimposed wave appears near the summit of the rapid ejection phase, causing a notched appearance. In the same animal, when the bevel was turned away from the heart, the superimposed wave is definitely lower on the ascending limb.

The middle pair of tracings in fig. 5, from animal 26, illustrates a change seen in 3 animals (12.5%) of the series. Here, with the bevel toward the heart, the superimposed wave on the ascending limb almost completely disappears.

In the bottom pair of tracings, from animal 11, little difference is seen whether the bevel of the needle be toward or away from the heart. Such was the case in 7 animals (29%) of the series.

In all cases, the upstream/downstream pulse pressures appeared to show only slight difference. Further, the general contour of the pulse wave remained the same whether the needle bevel was toward or away from the heart.

Thus, it appeared that conditions closely approximating lateral pressure recording were met, and that the main upstream/downstream effect of the bevel was upon the time relation and degree of registration of a superimposed natural vibration during the beginning of ejection.

However, it was decided routinely to

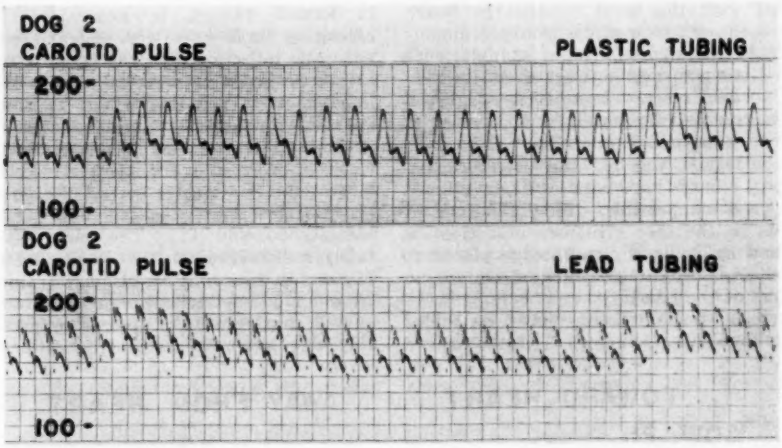


Fig. 3—Carotid pulse in nembutalized dog 2, comparing use of plastic and lead tubing. Note small waves superimposed on basic pulse contour, in lead-tubing record. Pulse pressures similar in both records. Note respiratory waves. Code marker at top margin denotes respiratory rate. (Code marker not vertically aligned with writing stylus.) Pressures at left margin in millimeters of mercury.

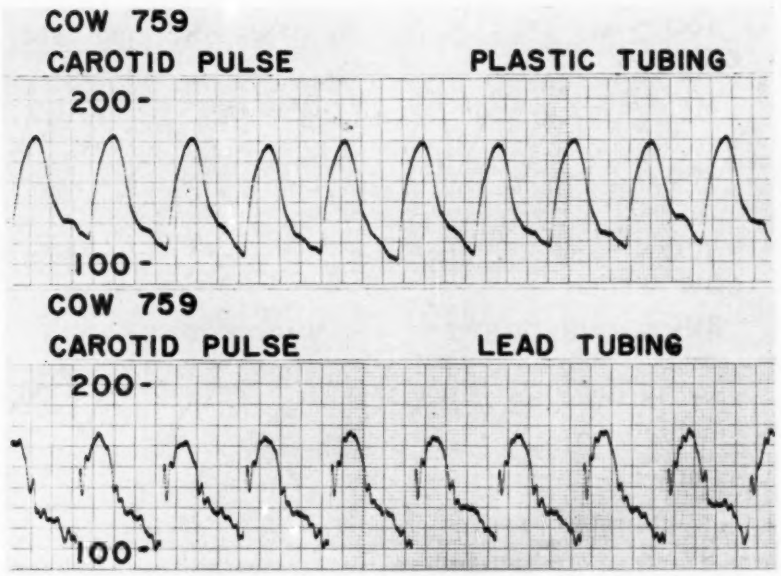


Fig. 4—Carotid pulse in dairy cow under local anesthesia, comparing use of plastic and lead tubing. Superimposed waves present in lead-tubing record. Pulse pressures and basic pulse contour similar in both records. Little respiratory variation. Code marker denotes respiratory rate. Pressures at left margin in millimeters of mercury.

record with the bevel toward the heart. The question of bevel position presumably could be obviated if a blunt cannula, such as a Lindeman needle, were used.

Arterial Pressure Recording.—Figure 6 shows records from animal A 63, which are typical of normal animals studied to date. The arterial, venous, and electrocardiographic records have been vertically aligned as closely as possible. Time relations of events in the three tracings may thus be studied by using a straightedge placed so as to cross all three tracings from top to bottom of the page.

Arterial pulse tracings have resembled in contour those described from the carotid in human beings, in general.[5, 11] The ascending limb was steep and showed an anacrotic inflection near the peak, in many instances (fig. 6, top record, a). A superimposed wave presumably due to free or natural vibrations set up in the periodic arterial system during the beginning of ejection, when the left ventricle ejects a large volume into a lax aorta,[5, 11] frequently deformed the ascending limb (fig. 5, animals 25 and 11). The incisura was fairly well marked on the descending limb (fig. 6, top record, b; *see* also fig. 5, animals 25 and 11). Postincisural waves were seen (fig. 6, top record, c) and were presumably due again to natural vibrations of

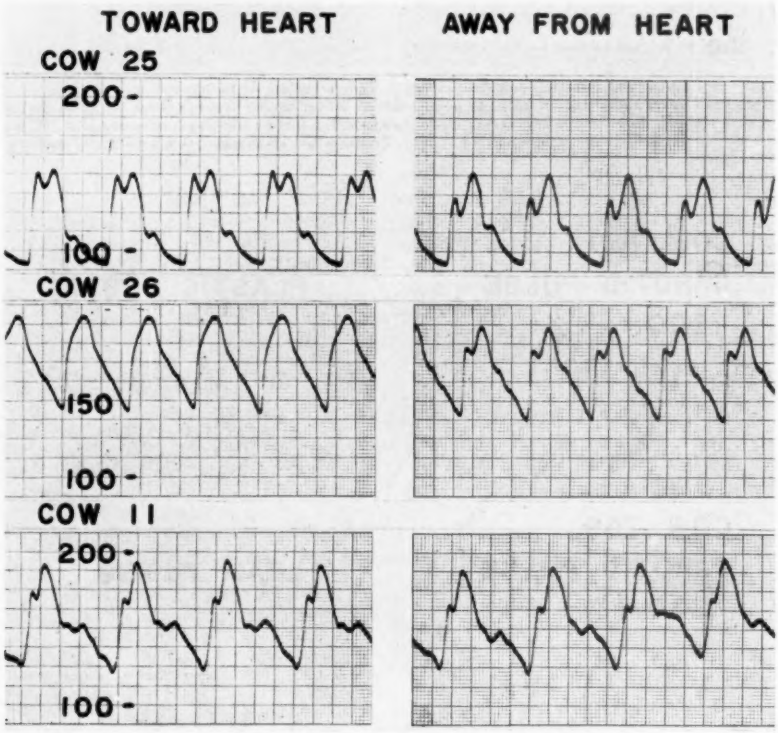


Fig. 5—Effect of bevel position on carotid pulse record in dairy cows.
Animal 25 (top pair of tracings): position of superimposed wave on ascending limb varies.
Animal 26 (middle pair of tracings): superimposed wave on ascending limb is marked only in downstream (bevel away from heart) record.
Animal 11 (bottom pair of tracings): little upstream/downstream difference in topographic features. Pulse pressures show only slight difference, general contour of pulse wave similar whether needle bevel is toward or away from the heart. Code marker denotes respiratory rate. Pressures at left margin in millimeters of mercury.

the arterial system set up upon closure of the semilunar valves.

Pressures recorded to date have in the majority of instances, in normal adult cows and heifers, been from 90- to 130-mm. mercury diastolic, 140- to 180-mm. mercury systolic, and 40- to 70-mm. mercury pulse pressure. These values are in general accord with those found by Götze[3] and Dukes,[2] using a sphygmomanometer and mercury manometer, respectively. Evaluation of the effects of age, pregnancy, and other factors is in progress.

Venous Pressure Recording. — Venous pulse tracings have been more difficult, as previously noted. They have shown fairly well-defined topographic features in some instances. Figure 6, middle record, shows a tracing in which the sequence of positive waves has been marked where most easily distinguishable. The "a" or presystolic wave is due to atrial contraction. The "c" or systolic wave is due to ventricular contraction and bulging of the atrioventricular valve into the atrium. Contributing also to the "c" wave may be a transmission of the pulse from the neighboring carotid artery. The "v" or diastolic wave reflects the rise in atrial pressure as that chamber is filled by the steady inflow of venous blood, the atrium being closed below by the atrioventricular valve. The "v" wave is thus due to the rise in atrial pressure preceding and the sudden

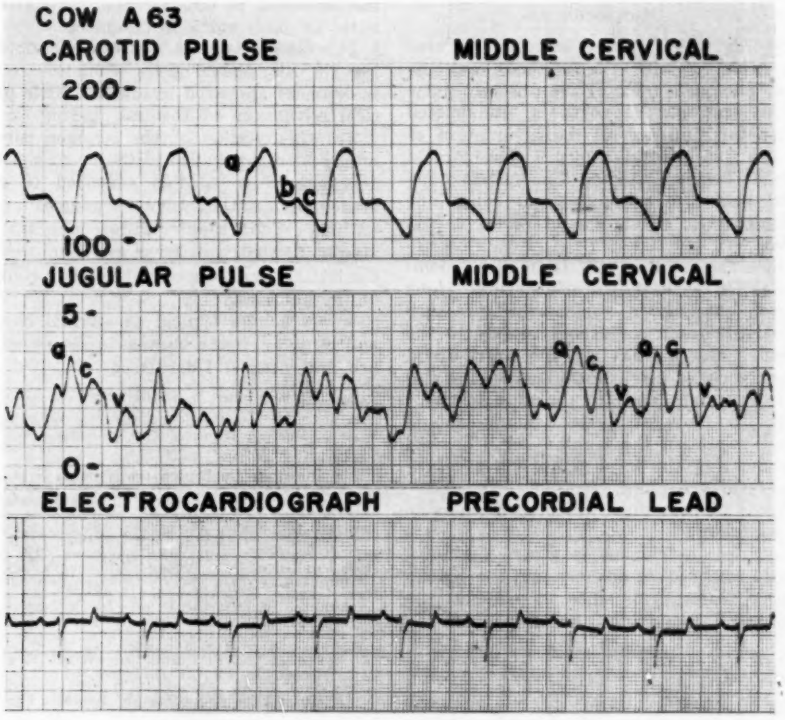


Fig. 6—Normal carotid and jugular pulses and electrocardiogram (precordial lead) in the dairy cow. The records have been vertically aligned as closely as possible.

Carotid pulse tracing: steep ascending limb, with anacrotic inflection, a; descending limb shows incisura, b; and postincisural waves, c, set up upon closure of semilunar valve.

Jugular pulse tracing: shows fairly well-marked wave sequence, including presystolic wave, a; systolic wave, c; and diastolic wave, v. Note modification of wave form and amplitude by respiration. Code marker denotes respiratory rate. Pressures at left margin in millimeters of mercury.

drop in pressure following opening of the atrioventricular valves.[4, 6, 11]

It will be noted in fig. 6 that respiration considerably modifies the wave form and amplitude. The carotid, jugular, and electrocardiographic tracings have been vertically aligned as closely as possible, as previously mentioned, to allow better appreciation of time relationship of events in the cardiac cycle and to provide general landmarks.

Mean jugular pulse pressures recorded to date in normal adult cows and heifers, measured in the middle cervical region, have ranged, in general, from 2- to 10-mm. mercury pressure. Maximum wave amplitudes have approximated 1- to 4-mm. mercury pressure.

Discussion

Records which appear satisfactory have been secured with the methods as outlined. The basic form of the pressure pulse in the arch of the aorta is transmitted rather faithfully up to the bifurcation of the common carotid artery.[11] The carotid tracing, besides yielding the systemic arterial pressures without great error due to hydrostatic effect, should reflect events in the left ventricle.

Since the jugular vein is in direct communication with the interior of the right atrium, changes in volume and pressure (relative) which correspond closely to the pressure fluctuations of the intra-atrial pressure curve are transmitted to the column of venous blood in the jugular vein.[1] Thus, the jugular pulse tracing should reflect events in the right atrium, as well as indicating the level of venous pressure at a reference point.

Points of procedure which seem valuable are as follows:

1) The instrument is given a ninety-minute warm-up in advance and the venous tracing taken first, since it presents the greater difficulty from the standpoint of the employment of a high level of sensitivity of the instrument for this measurement and the introduction of artifacts, such as those previously mentioned. The venous tracing is then followed by the arterial record and, finally, an electrocardiogram is taken, if desired.

2) After initial palpation of the structures concerned, cannulations are made without stasis in the case of the arterial cannulation, and with a minimum of stasis in the case of the venous cannulation. Hematomata, while not bothersome, could be kept at a minimum by this procedure. In the first series of 22 normal animals, the incidence of hematoma was as follows: marked, 1 (4.5%); moderate, 4 (18%); slight, 6 (27%). If hematoma formation occurred during or following cannulation, the vessels on the opposite side of the neck were used for measurement of the respective pressures. In no case, in the series mentioned, did infection occur at the site of cannulation, nor did the animals go off feed or milk production.

Summary

1) Application of electromanometric measurement to arterial and venous pressures in dairy cattle is described.

2) Comparison of lead and plastic tubing for connection of the cannulae to the instrument appeared to indicate little practical advantage of the lead tubing.

3) Cannulation of the common carotid artery at right angles to its long axis with 16-gauge, 3-in. needles appeared to give reasonably satisfactory pressure registration. Topographic differences in upstream/downstream bevel position records did not appear to involve the basic contour of the pulse wave.

4) Tracings typical of normal carotid and jugular pulse curves in dairy cattle are presented and discussed.

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The Occurrence and Significance of Agglutinins in the Genital Organs of Brucella-Infected Cows

AAGE JEPSEN and TORBEN VINDEKILDE

Copenhagen, Denmark

IN RECENT years, agglutination tests based upon the presence of agglutinins in the milk are being widely used in diagnostic programs for brucellosis in cattle. The fact that milk from infected cows sometimes will display higher agglutinin titers than blood serum has drawn attention to the problem of local formation of agglutinins in infected tissues.

Bendixen and Blom[1] found that in Brucella-infected bulls, with localization in the genital organs, the presence of agglutinins for *Brucella abortus* may be demonstrated in the seminal plasma, and that the agglutinin titer of the seminal plasma often is considerably higher than the corresponding titer of blood serum. Christensen[2] demonstrated high agglutinin titers in secretions and exudates pressed from infected ampullae ductus deferentes, glandulae vesiculares, and necrotic testes. The histologic picture of such tissues showed pronounced cell infiltrations, made up chiefly of plasma cells in close connection with glandular epithelium and demarcation membranes.

TABLE 1—Fourteen Cows with Positive Titer of Fluid from Uterine Mucosa

Cow	Titer		Bacteriologic examination
	Blood	FUM*	
3854	0	160	Brucella
6487	20	10	...
3 13/6	20	5	...
346	20	40	negative
3568	40	5	...
6520	40	5	...
5 13/6	40	5	...
341	40	10	...
3867	40	640	Brucella
2741	80	40	negative
4066	80	80	negative
3981	640	160	Brucella
4061	1280	640	negative
4256	1280	1280	negative

*FUM = fluid expressed from uterine mucosa. The figures are referred to as uterine titers. 0 means absence of any agglutination in dilution 1 : 5, the lowest dilution used. Titer figures indicate the highest dilution showing specific agglutination, total or partial. Bacteriologic examination means plating on tryptose-crystal-violet agar from ground uterine mucosa.

Materials and Methods

Investigations were conducted, along the same lines, of the genital organs of cows. At first, a number of cows delivered for slaughter were investigated. From each cow, agglutination titers of blood serum and of fluid from the uterine mucosa collected by freezing and thawing of the organ were examined. Results from examination of 39 cows are tabulated in tables 1 and 2.

TABLE 2—Twenty-Five Cows with Negative Titers of Fluid from Uterine Mucosa

No. of cows	Titer		Bacteriological examination
	Blood	FUM*	
1 cow	0	0	...
2 cows	5	0	...
6 cows	10	0	...
13 cows	20	0	...
2 cows	40	0	...
1 cow	640	0	...

*FUM = fluid expressed from uterine mucosa. The figures are referred to as uterine titers. 0 means absence of any agglutination in dilution 1 : 5, the lowest dilution used. Titer figures indicate the highest dilution showing specific agglutination, total or partial. Bacteriologic examination means plating on tryptose-crystal-violet agar from ground uterine mucosa.

Positive agglutination titers were displayed in fluid from the uterine mucosa of 14 cows, the titer varying from 5 to 1,280. In 1 case, no agglutinins were demonstrable in the blood and, in 2 other cases, uterine titers were higher than the corresponding blood titers. We succeeded in cultivating three strains of *Br. abortus* from the uterine mucosa.

Of 25 cows with negative uterine titers, 1 had negative blood titer, whereas 24 showed positive titers varying from 5 to 640.

Thus, having demonstrated that Brucella agglutinins may be present in fluid from the uterine mucosa of infected cows, we proceeded to investigate two large herds that recently had been freed from blood reactors but where reinfections had occurred. We thought it possible that the recurrence of reacting animals might be due to the presence in the herds of cows with a localized uterine infection, which had not given rise to any demonstrable blood reaction. Fluid for agglutination

From the Department of Bacteriology and Hygiene, Royal Veterinary and Agricultural College, Copenhagen, Denmark.

tests was collected from the genitalia of live cows as follows*: A sterile gauze tampon tied up with a string was deposited in the vagina and left there for one hour to absorb fluid from the genital canal. Usually, the tampon absorbed 3 to 4 Gm. of fluid. By means of the string, the gauze tampon was pulled from the vagina, transferred to a tube containing 6 cc. of saline, and left in the icebox overnight. Dilutions for the agglutination tests were made from the fluid pressed from the tampon. That fluid, we estimate, will correspond to a dilution of 1 : 2.5.

To transfer the tampon to the vagina, we employed sterile metal tubes, from which the tampon was pushed by means of a sterile rod, using a fresh tube for each cow.

Blood titers and uterine titers are compared in table 3.

TABLE 3—Comparison of Blood and Uterine Titers of Two Herds

	Herd B									
Cow	1	13	64	154	5	86	139	149		
Uterine titers 1-28-50	5	5	5	5	10	10	640	640		
Blood titers 1-19-50	<10	<10	<10		<10	10	800	200		
	Herd M									
Cow	8	19	68	77	129	151	171	173	149	185	163	192
Uterine titers 3-27-50	5	5	5	5	5	5	5	5	10	20	40	40
Blood titers 3-21-50	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	640

Herd B, with a total of 62 cows, was blood tested, Aug. 10, 1949. Four reactors were found and removed for slaughter. On Oct. 7, 1949, cow 149 aborted, and when retested on Jan. 19, 1950, she had developed a positive blood titer, as had also cow 139. On testing uterine titers nine days later, these 2 cows showed uterine titers of 640, and 6 additional cows showed uterine titers. One of these, (cow 13) aborted May 17, 1950 (*see* table 4). Herd M, with a total of 120 cows, had been free of blood reactors for about a year and a half. A milk ring test on a herd sample (March 17, 1950) gave a positive reaction which was followed up by a blood test on March 21, 1950. Three reactors were found. One was slaughtered immediately while the others were removed from the herd. On March 27, 1950, 1 of the blood reactors (cow 192) showed a uterine titer of 40 and was slaughtered without further examination. Another (cow 135) was found negative to the uterine test and calved April 7, 1950. No uterine infection with Brucella could be demonstrated. The uterine test, on March 27, 1950, disclosed 11 cows with positive uterine titers and negative blood titers. One of these, cow 151, aborted May 13, 1950 (*see* table 4). Cows 185 and 163 are pregnant and are still being held for observation. Both have now developed positive blood reactions.

TABLE 4

Cow 13, herd B, born 1946	Date	Titers: Blood	Titers: Uterine	Milk ring test
	8-10-49	<10	...	...
	9-22-49	...	...	±
	1-2-50	...	...	±
	1-19-50	<10	...	...
	1-28-50	...	5	...
	5-8-50	100	...	...
Abortion 5-17-50, Brucella demonstrated				
	5-19-50	640	1280	+++
	6-2-50	2560	2560	+
Cow 151, herd M, born 1944	3-12-47	<10	...	...
	9-23-47	<20	...	...
	3-31-48	<20	...	...
	5-13-48	<20	...	...
	10-16-48	<20	...	...
	3-30-49	<20	...	...
	10-3-49	<20	...	...
	3-21-50	<20	...	...
	3-27-50	...	5	...
	4-14-50	...	...	++
	4-27-50	80	...	+++
	5-12-50	320	20	+
Abortion 5-13-50, Brucella demonstrated				
	5-19-50	1280	160	+++
	5-26-50	1280	10240	++

Herd B, with a total of 62 cows, was blood tested, Aug. 10, 1949. Four reactors were found and removed for slaughter. On Oct. 7, 1949, cow 149 aborted, and when retested on Jan. 19, 1950, she had developed a positive blood titer, as had also cow 139. On testing uterine titers nine days later, these 2 cows showed uterine titers of 640, and 6 additional cows showed uterine titers. One of these, (cow 13) aborted May 17, 1950 (*see* table 4). Herd M, with a total of 120 cows, had been free of blood reactors for about a year and a half. A milk ring test on a herd sample (March 17, 1950) gave a positive reaction which was followed up by a blood test on March 21, 1950. Three reactors were found. One was slaughtered immediately while the others were removed from the herd. On March 27, 1950, 1 of the blood reactors (cow 192) showed a uterine titer of 40 and was slaughtered without further examination. Another

When cow 151 was slaughtered eighteen days after it had aborted, fluid from the uterine mucosa was collected, as described, for our slaughterhouse material. The agglutination titer was 40,960.

CONCLUSION

Investigations so far have demonstrated that a local formation of agglutinins may take place in the genital organs of Brucella-

*This method was first used to detect *Vibrio fetus* infection by L. Szabo.

infected cows. The agglutination titers of fluid from the uterine mucosa in several cases were higher than the corresponding blood titers. A technique for collecting fluid for agglutination test from the vagina of the cow was developed and, in two herds, a number of cows were found with negative blood titers but positive uterine titers. Two of these cows later developed positive blood reactions and aborted. After abortion, very high uterine titers developed.

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Pathology of Eastern Equine Encephalomyelitis

R. E. KISSLING, D.V.M., and HARRY RUBIN, D.V.M.

Montgomery, Alabama

THE INFECTIOUS equine encephalomyelitides are of agricultural and public health importance. Due to the overlapping of the geographic areas involved, the differential diagnosis of the eastern and western strains of equine encephalomyelitis must depend largely upon laboratory procedures. The object of this study was to examine brains of horses dead with eastern equine encephalomyelitis to add to the information on the pathology produced by these virus infections. Such information would prove valuable in those cases where virus-isolation techniques fail or death of the animal precludes the obtaining of satisfactory serum samples. A detailed pathologic study has not been made previously with equine material infected with the eastern strain of virus.[1]

Studies on human material[2, 3, 4, 5, 6] have suggested that the lesions produced by western equine encephalomyelitis virus are both destructive and proliferative in nature, while those produced by the eastern strain are primarily vascular in character and show large numbers of hematogenous cells infiltrating the brain substance. Differential studies using the usual laboratory animals, in general, have been rather nonproductive.[7, 8, 9]

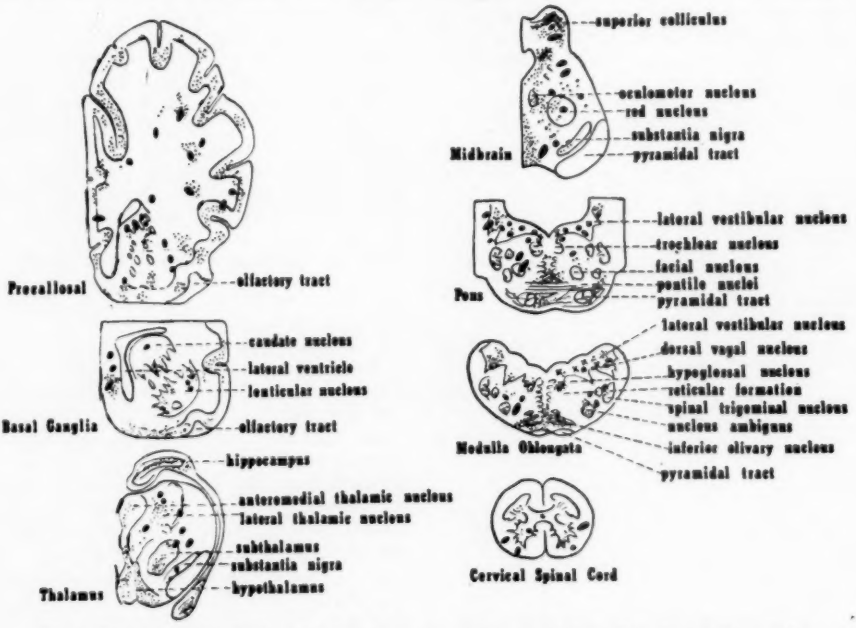

Fig. 1—Topographic distribution of lesions in eastern equine encephalomyelitis. Dots indicate cellular infiltrations; crosses, hemorrhage; and solid black circles, perivascular infiltrations.

MATERIALS AND METHODS

Brains were collected from horses and mules affected during the 1948 and 1949 epizoötics of eastern equine encephalomyelitis in Georgia,

From the Laboratory Services, Virus and Rickettsia Section, Communicable Disease Center, Montgomery, Ala.

Louisiana, and Arkansas. Seven of the specimens were complete except for portions removed for virus isolation. Two consisted of one half of the brain anterior to the anterior colliculus. Selected blocks of 12 additional cases were submitted by Dr. L. E. Starr of the Georgia State Health Department. The tissues were fixed in buffered 10 per cent formalin, chromated, embedded in paraffin, sectioned at 6 μ, and stained with hematoxylin-azure-eosinate. Selected blocks were frozen, sectioned, and stained by Penfield's combined method for microglia and oligodendroglia, Bielschowsky's method for neurofibrils, and by Graham's *alpha*-naphthol-pyronine stain for oxidase granules. Other paraffin sections were stained for myelin by Lillie's variant of the Weil-Weigert's method, and with periodic acid-leucofuchsin to demonstrate Schiff-positive material. Approximately, 40 to 45 blocks were taken from each of the complete brains, 20 from the cerebral cortex, four from the basal ganglia, two from the thalamic region, two from the midbrain, four from the cerebellum, four from the pons, five from the medulla oblongata, and one from the cervical cord.

The virus of eastern equine encephalomyelitis was isolated from 17 of the 21 specimens. Two of those in which virus was not isolated showed significantly high serum-neutralization indexes. Serum samples were not available on the remaining 2 cases.

NATURE OF THE LESIONS

The types of lesions fall into two general groups, with a few specimens showing intermediate stages.

In those animals surviving one day or less following the onset of nervous symptoms, there was a diffuse infiltration of the gray matter with polymorphonuclear leucocytes. Occasionally, ill-defined foci of denser accumulations were formed. These phagocytic cells contained fairly large amounts of glycogen, as demonstrated by the periodic acid-leucofuchsin routine with

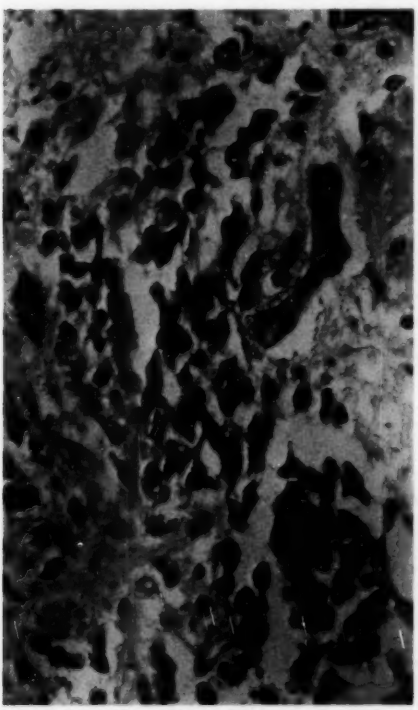

Fig. 2—Horse 578. Cerebral cortex showing diffuse infiltration by polymorphonuclear leucocytes. Duration, one day. Hematoxylin-azure-eosinate stain. x 600.

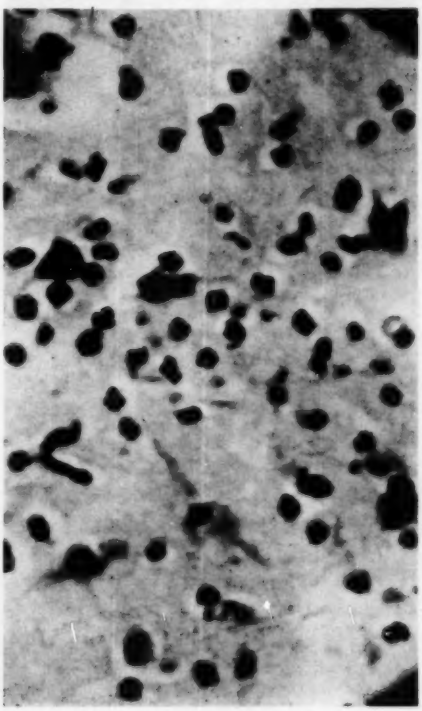

Fig. 3—Horse 571. Microglial nodule in hypothalamus. Duration, two days. Hematoxylin-azure-eosinate stain. x 430.

diastase-digested controls. The polymorphonuclear leucocytes were identified, in addition to their morphology, by the presence of peroxidase granules.

The vascular changes in these peracute cases consisted of severe endothelial damage, as shown by the formation of thrombi and swelling of the endothelial cells. The adventitia and the Robin-Virchow's spaces contained an exudate composed of approximately equal numbers of lymphocytes and polymorphonuclear leucocytes. Both the venules and arterioles showed these changes, with the venules possessing more severe lesions. The exudate did not spill into the surrounding parenchyma out of the Robin-Virchow's spaces. Perivascular hemorrhage and moderate perivascular edema were common. The capillaries and reticulum of the vessel walls were only weakly Schiff-positive. The reticulum appeared swollen and fragmented. Neuron damage in all cases was surprisingly mild compared to the inflammatory changes. There was some swelling of the neurofibrils. Occasionally, there was margination of the Nissl's substance, less often complete loss of the Nissl's substance, and rarely frank necrosis. Neuronophagia was seen only very rarely. Most of the neurons were anatomically normal.

In animals surviving two days or longer, polymorphonuclear leucocytes were no longer demonstrated and were replaced by both nodular formations and diffuse infiltrations of microglia. In silver carbonate preparations, these cells showed mobilization as characterized by retraction of their dendritic processes to form a more compact cytoplasmic mass. "Gitter" cells, however, were not demonstrated. The nodules con-

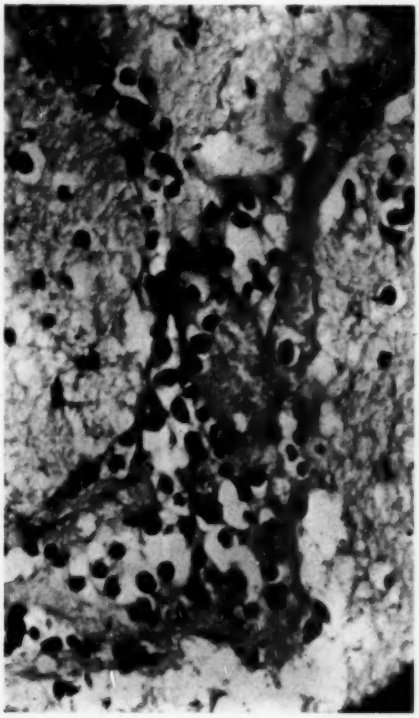

Fig. 4—Horse 578. Blood vessel in piriform lobe showing acute damage to endothelium. Hematoxylin-azure-eosinate stain. x 430.

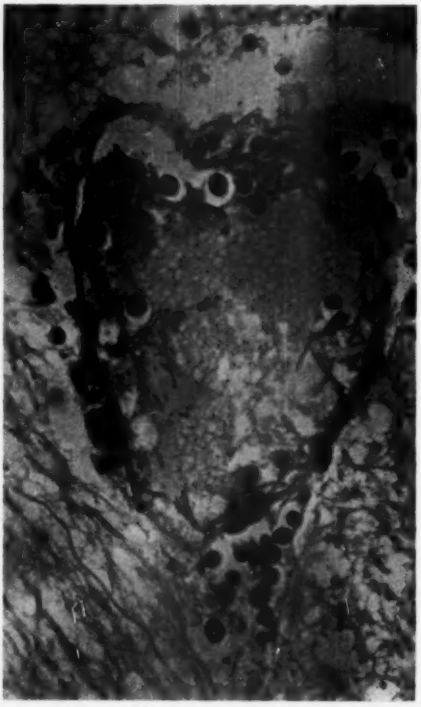

Fig. 5—Horse 578. Blood vessel in pontile tegmentum showing thrombus. Hematoxylin-azure-eosinate stain. x 430.

tained large mononuclear cells in addition to the microglia.

The perivascular exudate at this stage was almost exclusively lymphocytic. The endothelial proliferation in small vessels was, at times, extensive enough to give the impression of a nodule composed of large mononuclear cells.

Intermediate stages, in which there was a thinning of the polymorphonuclear leucocytic infiltrate and a mobilization of the microglia, were seen in 2 cases dying during the second day of symptoms.

The leptomeningeal reaction was mild and was chiefly vascular, resembling those vessels deeper in the brain substance.

Myelin techniques and fat stains showed no abnormality in the white matter.

No inclusion bodies were observed.

DISTRIBUTION OF THE LESIONS

The damage was limited almost exclusively to the gray matter, with only occasional foci of polymorphonuclear lymphocytes or microglia located in the white matter.

The cerebral cortex showed severe damage in all areas. Although there was no apparent predilection for any particular type of cortex, the frontal and occipital poles were more severely affected than the rest. The piriform lobe was less involved than the remainder of the cortex. Denser accumulations of the inflammatory cells were present in those portions adjacent to the deeper parts of the sulci than at the crests of the gyri. The cortex of the substantia perforata anterior was severely affected in a few cases and only moderately so in the remaining ones. The uncus and hippocampus, in all cases, showed heavier damage than the neocortex.

In general, the corpus striatum showed mild involvement consisting chiefly of perivascular cuffing. Parenchymal damage was usually more severe in the gray stripes between the caudate nucleus and putamen than in the body of either structure. Periventricular infiltration was usually present.

The lateral thalamic nucleus showed more severe damage than the anterior or dorsomedial nuclei, in all except 2 cases in which the reverse was true. Likewise, the lateral hypothalamic nucleus was more severely involved than the medial nuclei or the mammillary bodies. The subthalamic nucleus was uniformly the seat of relatively heavy infiltrations of reacting cells.

In the midbrain, an apparently random distribution was present, no particular predilection being shown for the oculomotor nucleus, red nucleus, or substantia nigra. A single large focus of inflammatory cells often would involve several functional units. The colliculi usually showed heavier damage than those structures immediately adjacent or ventral to the cerebral aqueduct.

The cerebellum showed less involvement than any other portion. The damage was limited chiefly to the roof nuclei and the cortex of the vermis.

The lesions in the brain stem, while located principally in the gray matter, gave no impression of involving any particular nerve pathways or systems. In general, the motor nuclei showed heavier damage than the sensory groups, the principal excep-

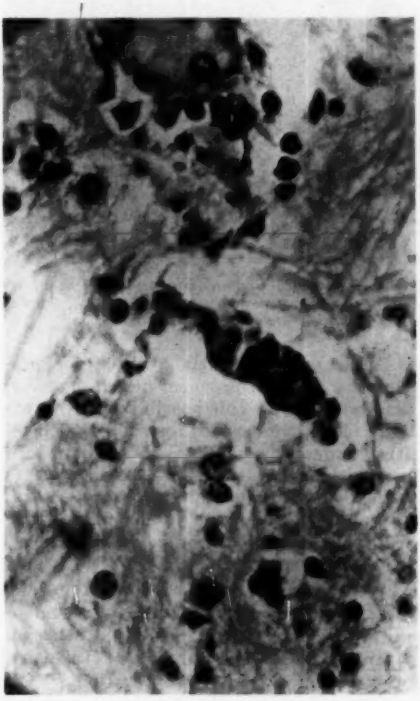

Fig. 6—Horse 571. Blood vessel in medulla oblongata showing occlusion by swollen endothelium. Hematoxylin-azure-eosinate stain. x430.

tion being in the vestibular nuclei which were damaged heavily in all cases.

The cervical cord showed much less damage than any level of the medulla oblongata.

DISCUSSION

Due to the fact that both infiltrating hematogenous cells and proliferating microglia are seen in fatal equine cases of eastern equine encephalomyelitis, the presumptive basis upon which human material (eastern strain) can possibly be differentiated from western equine encephalomyelitis can not be used on horse material.* Instead, the nature of the lesion appears to be related to the time factor, the early cases showing hematogenous cells which are replaced at a later stage by microglia.

Fig. 7—Horse 578. Frontal cortex showing heavy infiltration by polymorphonuclear leucocytes. Hematoxylin-azure-eosinate stain. x 100.

The olfactory pathways were uniformly severely affected. However, due to the widespread damage and random involvement of all parts, an infection through neuronal pathways appears improbable. Likewise, there is no evidence of the infectious agent being disseminated by cerebrospinal fluid. The most probable means of introduction of the agent into the brain is through the vascular system. This is dependent upon the presence of a "viremia" during some stage of the disease.

Because of the large numbers of infiltrating inflammatory cells in spite of the apparent minimal neuron damage, it is suggested that the involvement within the central nervous system is at the synaptic junctions instead of in the cell body or that the acuteness prevents the development of obvious neuronal changes. The pantropic properties of this virus are emphasized by the severe vascular damages observed.

Clinicopathologic Correlation.—The staggering, imbalance, and tendency to circle among the early symptoms of encephalomyelitis probably are associated with lesions in the vestibular nuclei. The morbid, stuporous somnolence frequently associated with this disease may be correlated with the irritative lesion in the hypothalamic nuclei, thus increasing the known suppressor action of these centers on cortical stimulation. The spasticity usually evident in the later stages of the disease may be attributed to release of the lower motor neurons from upper motor neuron regulation following severe damage to the cerebral cortex. The generalized convulsive seizures seen in the final stages of the disease are manifestations of irritation to the motor neurons of the cortex. The immediate cause of death is probably respiratory and circulatory failure following involvement of the respiratory and cardiovascular centers in the reticular formation of the medulla.

SUMMARY

1) Fatal cases of eastern equine encephalomyelitis are characterized by severe widespread involvement of the gray matter of the brain.

2) In early cases, the infiltrating cell is hematogenous, replaced by microglia during later stages.

3) The spread through the brain is apparently by means of the blood stream. Heavy vascular damage is present.

4) No area of the brain entirely escapes damage, however, the cerebral cortex, hippocampus, hypothalamus, and dorsal

*Studies on human material suggest that there is a difference in the pathologic changes produced by the two strains of virus. However, some pathologists feel that this apparent difference is due only to the variation in acuteness of the disease process produced by the respective strains.

nuclei of the medulla uniformly show the most severe damage.

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Antibody Response of Horses to Japanese Equine Encephalomyelitis Mouse Brain Vaccine

MAJOR KENNETH F. BURNS, V.C., U.S. ARMY

WITH THE isolation, in mice, of the virus of Japanese equine encephalomyelitis and subsequent studies related thereto,[1,2] it was the author's considered opinion that this agent was immunologically identical with the virus of Japanese B encephalitis isolated from human beings.[3] Kii *et al.*[4, 5] studied the problems of vaccination of equine animals against this disease. They immunized horses with a formolized mouse brain vaccine and challenged the vaccinated animals intracerebrally. Inoculated horses withstood the challenge dose, while control animals, which were simultaneously infected with the virus, fell victims to the disease.

A field trial to test the value of Japanese equine encephalomyelitis vaccine prepared by the author was undertaken to determine the sero-immunologic response of horses following vaccination with this type of biological product.

MATERIALS AND METHODS

Horses.—In order to obtain nonimmune horses for this experiment, 36 animals stabled on farms in Tokyo and Chiba prefectures were tested for neutralizing antibodies against the Nakayama strain of Japanese B encephalitis. Two serum specimens were obtained from each horse on Jan. 15 and 29, 1948. The results of neutralization tests are shown in table 1. A duplication of all results was observed in each of the two tests, and 26 of the negative horses were selected for experimental purposes.

Vaccine.—A group of mice were inoculated intracerebrally with the virus of Japanese equine encephalomyelitis, strains 2683 and 2869. These neurotropic agents had been isolated from brain tissues of fatal cases succumbing to this infection during the 1947 epizoötic.[6] When the inoculated mice were moribund, the brain tissues were removed and ground in a Waring blendor to a 10 per cent suspension, using saline as a diluent. To the suspension was added formalin to a final concentration of 0.2 per cent. The formolized brain emulsion was incubated overnight at room temperature and then deposited in an electric refrigerator until the virus was found to be inactive as exhibited by mice upon intracerebral inoculation with the undiluted vaccine. Controls, including safety and sterility tests, aërobic and anaërobic, were negative. The vaccine was now ready for use. Storage temperatures for the vaccine during this experiment were maintained at 2 to 5 C.

TABLE 1—Preliminary Tests for Neutralizing Antibodies to the Virus of Japanese B Encephalitis (Nakayama) on Blood Serums of Horses

Prefecture	Age	Results of neutralization tests*			
		Positive	Equivocal	Negative	Total
Chiba	1 yr.	1	0	11	12
Tokyo	1 yr.	2	0	6	8
	2 yr.	0	0	2	2
	3 yr.	5	0	9	14
Total		8 (22%)	0	28 (78%)	36

*Neutralization tests: positive = neutralization index 50 or greater; equivocal = neutralization index 10 to 49; negative = neutralization index less than 10.

Equal volumes of the vaccine which were prepared from the two different strains were combined for the second and third antigenic stimuli. The $l.d._{50}$ (immunogenic dose) determination of these two lots of vaccine, 1 and 2, is shown in table 2.

TABLE 2—Potency Tests, in Mice, for Japanese Equine Encephalomyelitis Formolized Mouse Brain Vaccines 1 and 2

Vaccine	$l.d._{50}$ of vaccine when challenged with		
	Nakayama	2683	2869
1(2683)	0.0030 (8.0)*	.....	0.0021 (7.3)
1(2869)	0.0024 (8.0)	.....	0.0012 (7.3)
2(2683 and 2869)	0.0012 (8.1)	0.0035 (8.3)	0.0012 (8.0)

*Figures in parentheses represent –log (intracerebral $l.d._{50}$) of challenge virus used in potency tests.....=potency test not conducted.

The immunogenic potency test of the vaccine was conducted in accordance with the procedures as outlined by the Federal Security Agency, National Institutes of Health, minimum requirements for Japanese B encephalitis vaccine, revision of Feb. 5, 1945.

Schedule of Vaccination.—As shown in table 3, equine animals in Tokyo and Chiba prefectures were divided into groups A and B. Group A received subcutaneously three doses of 2, 2, and 4 ml. each of vaccine, while group B was

From the Department of Virus and Rickettsial Diseases, 406th Medical General Laboratory, APO 500, c/o Postmaster, San Francisco, Calif.

inoculated in the same manner with three doses of 4, 4, and 8 ml. of brain tissue vaccine. Those animals in the Tokyo group received their second and third immunizing doses at intervals of eight and eleven days, while those of the Chiba group were immunized at intervals of ten and eleven days.

similar manner. These antigenic preparations were used undiluted and had at least four to eight units of antigen in the 0.25-ml. amounts used in the tests. All equine serums were inactivated at 60 C. for twenty minutes.

Along with those antigens previously mentioned were two other diagnostic agents which had been prepared from the Nakayama strain and western equine encephalomyelitis (WEE) virus by benzene extraction and subsequent lyophilization, in accordance with the technique outlined by Espana and Hammon.[5, 6]

TABLE 3—Vaccination Schedule of Horses Against the Virus of Japanese Equine Encephalomyelitis

	Tokyo	Chiba	Group A	Group B
1st vaccination and bleeding	2-25-48	2-24-48	2 cc., vaccine 1, subcutaneously	4 cc., vaccine 1, subcutaneously
2nd vaccination and bleeding	3- 4-48	3- 5-48	2 cc., vaccine 2, subcutaneously	4 cc., vaccine 2, subcutaneously
3rd vaccination and bleeding	3-15-48	3-16-48	4 cc., vaccine 2, subcutaneously	8 cc., vaccine 2, subcutaneously
4th bleeding	3-29-48	3-30-48	.....	.....
5th bleeding	5-27-48	5-28-48	.....	.....

Neutralization Tests.—Blood specimens were collected from the jugular vein in the usual manner and, following separation of the serums, were placed in an insulated chest containing solid CO_2 and were frozen. The serums remained in this state until just prior to testing. Intracerebral neutralization tests were carried out according to the technique recommended by the Commission on Virus and Rickettsial Diseases.[6]

In this study, two strains of the virus, Nakayama and 2683, were used simultaneously for the neutralization tests. The criteria of results are: no serum is regarded as positive unless the neutralization index is 50 or greater, those less than 10 are negative, and serums portraying indexes between 10 and 50 are expressed as equivocal.

Complement-Fixation Tests.—This test was carried out according to the procedure described by Casals and Palacios.[7] The antigens for use in this test were prepared by the high speed centrifugation method[8] from the Nakayama strain of Japanese B encephalitis and the two equine isolates, 2683 and 2869. A normal mouse brain-control antigen was prepared in a

RESULTS AND DISCUSSION

Following vaccination, complications for the most part were mild, consisting of local induration, edema, pain, and tenderness. These symptoms were slight and transitory in nature. Systemic reactions were observed in only 2 horses. These animals exhibited a slight rise in temperature without any other signs of disorder on the day following the initial dose of vaccine. Subsequent doses were tolerated with no systemic reactions being presented.

The results of neutralization tests in groups A and B on the serums of vaccinated horses, in Tokyo and Chiba prefectures, are shown in table 4. This summarization clearly demonstrates the

TABLE 4—Combined Results of Neutralization Tests of Tokyo and Chiba Horses Vaccinated with Mouse Brain Vaccines 1 and 2

Group	Neut.**	1st bleeding		2nd bleeding		3rd bleeding		4th bleeding		5th bleeding	
		Naka.*	2683*	Naka.	2683	Naka.	2683	Naka.	2683	Naka.	2683
A***	+	0	0	2	4	4	5	5	6	13	12
	±	0	0	1	4	1	1	6	2	0	1
	—	15	15	12	7	10	8	1	4	1	1
Total		15	15	15	15	15	14	12	12	14	14
B***	+	0	0	1	2	2	5	4	4	10	10
	±	0	0	0	1	1	1	6	5	0	0
	—	11	11	10	8	8	4	1	2	1	1
Total		11	11	11	11	11	10	11	11	11	11
A and B	+	0	0	3	6	6	10	9	10	23	22
	±	0	0	1	5	2	2	12	7	0	1
	—	26	26	22	15	18	12	2	6	2	2
Total		26	26	26	26	26	24	23	23	25	25

*The strains which were used in the neutralization tests. **Neutralization index: see footnotes to table 1. ***Group A received three doses of 2, 2, and 4 cc. each of vaccine. Group B received three doses of 4, 4, and 8 cc. each of vaccine.

influence of the potency of the vaccine. Neutralizing antibodies were exhibited against both strains of the virus (Nakayama and 2683).

No significant difference in immunogenic response was observed between groups of horses A and B, although group B received twice the volume of vaccine. Group A (table 4), which received a total vaccine dosage of 8 cc., showed that 13 and one of 14 serums on final bleeding were positive and negative, respectively, to the Nakayama strain, while 12, one, and one of the same group were positive, equivocal, and negative, respectively, to strain 2683. In group B (table 4), 10 and 1 of 11 horses were positive and negative to the Nakayama and 2683 strains.

As expected, the number of horses which were positive for antibodies increased as the schedule of vaccination progressed; thus, in the combined data, 11.5, 23.1, 39.1, and 92.0 per cent were positive to the Nakayama strain at second, third, fourth, and fifth bleedings, respectively. Strain 2683 exhibited positive titers on identical bleedings, having 23.1, 41.7, 43.4, and 88.0 per cent of serums positive.

The final bleeding, carried out seventy-three days following the last dose of vaccine, showed that the serums of 92.0 per cent of all horses were positive for neutralizing antibodies.

Some difference was observed between the results of neutralization tests using the Nakayama and 2683 strains, suggesting antigenic dissimilarity between these two strains of virus. Because of the inherent inaccuracies of this test, which are produced by a number of uncontrolled variables, we are unable to confirm this opinion.

Complement-fixing antibodies appeared in all vaccinated horses. Cognizance must be taken of the fact that appreciable amounts of complement-fixing antibodies for the normal mouse brain factor were demonstrated in addition to the antigens of Nakayama, 2683, and 2869 strains. Detailed representative results are shown in table 5. In a majority of the horses, the difference in titer was either negative or slight between the antigens prepared from infected brains and the normal mouse brain control. In 4 illustrative cases, 1 of them, Kyokushu, a typical response, there was no significant increase in titer over that of the control (table 5). These results were also confirmed in the tests using antigens which had been prepared by the benzene-extraction method. In this test, antigens of Nakayama and

TABLE 5—Complement-Fixation Response of Horses to Japanese Equine Encephalomyelitis Mouse Brain Vaccine

Prefecture	Name of horse	Titer of complement-fixation tests against the indicated antigen					
		Serum Bleeding	Nakayama*	2683*	2869*	NMB*	Nakayama**	WEE**
Tokyo	Kyokushu	1st	0***	0	0	0	....	....
		2nd	1:8	1:4	1:16	1:4	1:8	1:4
		3rd	1:32	1:16	1:32	1:8	1:32	1:8
		4th	1:64	1:32	1:32	1:8	1:64	1:8
		5th	1:4	0	0	0	....	....
Tokyo	Fujii	1st	1:2	1:2	1:4	1:2	1:2	0
		2nd	1:16	1:4	1:16	1:8	1:16	1:4
		3rd	1:8	1:4	1:8	1:4	1:16	1:16
		4th	1:16	1:16	1:32	1:32	1:32	1:16
		5th	1:4	1:4	1:4	1:4	....	....
Tokyo	Kinshi	1st	1:8	1:2	1:4	0	....	....
		2nd	1:64	1:64	1:64+	0	1:16	0
		3rd	1:128	1:128	1:128	0	1:64+	0
		4th	1:128***	1:256	1:128	1:8	1:64+	1:16
		5th	1:64	1:32	1:32	0	....	....
Chiba	Nishiki	1st	1:2	1:2	1:2	1:2	....	....
		2nd	1:16+	1:16+	1:16+	1:16+	1:16+	1:16+
		3rd	1:16	1:16	1:16	1:16	1:32	1:32
		4th	1:8	1:16	1:16	1:8	1:16	1:16
		5th	1:4	0	1:4	0	....	....
Chiba	Hoichi	1st	0	1:2	1:2	1:2	....	....
		2nd	1:16+	1:16+	1:16+	1:16+	1:16+	1:16
		3rd	1:16	1:32	1:16	1:8	1:32	1:16
		4th	1:16	1:32	1:16	1:8	1:32	1:16
		5th	1:8	1:8	1:4	1:4	....	....

*Antigens prepared by high speed centrifugation. (NMB = normal mouse brain.) **Antigens prepared by benzene extraction. (WEE = western equine encephalomyelitis.) ***The figures refer to the highest dilution of serum producing the fixation of complement.....=test not conducted.

WEE were used. The results with WEE antigens were positive in addition to those for Japanese B antigen. The positive reaction with WEE antigen was not considered to be specific for the virus of WEE but was due entirely to the normal mouse brain component present in the antigen employed.

In table 5, data on the complement-fixation tests of the horse, Kinshi, are shown which were not included in the experiment proper because of the presence of neutralizing antibodies on preliminary tests. Kinshi received three doses of 4, 4, and 8 ml. each of the vaccine as did the members of group B. This horse demonstrated a low titer of complement-fixing antibodies for Nakayama, 2683, and 2869 antigens prior to vaccination, and produced specific complement-fixing antibodies for the equine and human viruses in a high titer eight days following the initial dose of vaccine. Complement-fixing antibodies for the normal mouse brain control on this animal were not present in a 1 : 2 dilution of the serum; later they appeared, although the titer was low in comparison with that against the virus antigen. These results were marked in contrast to the horses which had no neutralizing and complement-fixing antibodies prior to vaccination. This phenomenon was considered to be a specific reaction. Similar results were obtained in human beings by Sabin *et al.*[10] They reported that complement-fixing antibodies specific for the virus of Japanese B encephalitis appeared rapidly in those individuals (10 aged people) who had neutralizing antibodies prior to inoculation of the Japanese B vaccine (evidence of previous inapparent infection) but in none of the 9 aged people who were without such antibodies.

SUMMARY

Twenty-six horses which had negative neutralization indexes to the virus of Japanese B encephalitis were vaccinated subcutaneously with a formolized vaccine. This biological product was prepared from mouse brains infected with the virus of Japanese equine encephalomyelitis. These horses developed neutralizing antibodies to the viruses of Japanese B encephalitis and Japanese equine encephalomyelitis following their vaccination.

Eighty-eight per cent of the inoculated animals showed a positive neutralization index to the virus of Japanese equine encephalomyelitis.

Based upon the premise that circulating neutralizing antibody will afford protection against natural infection, this type of biological product may offer protection against Japanese equine encephalomyelitis as it occurs naturally.

Complement-fixing antibodies for the normal mouse brain component were regularly produced and, in some instances, part of the rise appeared to be specific for the virus. Following vaccination, only 2 of the 26 horses demonstrated a significant and possibly specific increase in complement-fixing titer to the virus of Japanese B encephalitis and Japanese equine encephalomyelitis.

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Screening Tests of Iodine Compounds Against Horse Strongyle Larvae

NORMAN D. LEVINE, Ph.D.

Urbana, Illinois

THE USE of horse strongyle larvae for the systematic testing of chemicals for nematocidal activity was introduced by Parnell.[4] His method was also employed by Levine[2] for testing 70 compounds. A review of the literature is given in the latter paper.

Cameron and Parnell[1] reported that zinc iodide (0.05%), potassium iodide (0.06%), sodium iodide (0.06%), potassium iodate (0.1%), and ferrous iodide (0.12%), when mixed with horse feces, prevented the development of the great majority of horse strongyle larvae. Parnell[5] reported that methyl iodide (0.01%), iodoform (0.1%), and iodine (0.25%) were effective. Levine[2] found that potassium iodide (0.1%) or sodium iodide (0.1%) prevented the development of all larvae; 0.01 per cent of these compounds prevented the development of almost all larvae. Iodoform also prevented the development of all larvae in a concentration of 0.1 per cent but, in a concentration of 0.01 per cent, about half the larvae developed.

The present paper gives the results obtained with 61 iodine compounds.

MATERIALS AND METHODS

The technique used in this study, a modification of that used previously,[4,2] has been described by Levine.[3] Each compound was mixed with talcum powder to give it bulk, and 0.5 Gm. of this mixture was mixed further with 4.5 Gm. of fresh horse feces. This mixture was placed on a double layer of cheesecloth, which was made into a bag by tying its ends around the sample, and the bag was then suspended by the string in a closed, 2-oz., wide-mouth glass jar. The jar was placed in a dark cupboard for seven days at room temperature and then examined. By this time, the horse strongyle larvae present had molted twice and reached the third, infective stage. Many had migrated out of the feces and could be seen with a high-power hand lens in the droplets of condensation water present on the sides of the jar.

If no larvae were seen, the bag was placed in a funnel fitted with rubber tubing closed by a pinch-clamp. Warm water was added to partially cover the bag, and allowed to stand for an hour or two. Some of the water was then withdrawn from the tube at the bottom of the funnel and examined for larvae with a hand lens or low-power microscope.

Untreated controls were set up at the same time as the treated samples to make sure that larvae would have developed normally.

The presence of moderate to abundant larvae in a jar indicated that the compound was ineffective against the eggs and/or larvae. Occasionally, a very few larvae were found in a test. Since these represented less than 1 per cent of the normal number of larvae, the compound was considered effective in these cases.

In the preliminary screening test, each compound was set up in concentrations of 0.01, 0.005, 0.0025, 0.001, 0.0005, and 0.00025 M.* If promising results were obtained at these concentrations, further studies were made at lower concentrations. Some of the compounds which were ineffective in a concentration of 0.01 M. were tested at higher concentrations.

TABLE I—Effect of Inorganic Iodine Compounds on Horse Strongyle Larvae in Manure

Compound	Lowest lethal concentration	
	Molar	Per cent
Ammonium iodide	0.00025	0.0036
Mercuric iodide (red)	0.00025	0.0110
Potassium iodide	0.00050	0.0080
Sodium iodide	0.00025	0.0038
Potassium iodate	0.00050	0.0110
Potassium metaperiodate	0.00050	0.0120

Manure was obtained from 5 horses in the University of Illinois herd. Strongyle egg counts varied from 750 to 9,600 eggs per gram. Of 2,200 larvae identified from the horses dur-

From the College of Veterinary Medicine and Agricultural Experiment Station, University of Illinois, Urbana.

The technical assistance of Walter Fehrenbacher is acknowledged.

*Comparisons are made on a molar rather than a percentage basis because the latter is misleading. In comparing the activity of different compounds, differences between equal numbers of molecules are more significant than differences between equal weights of the compounds. For example, a comparison of ammonium iodide with mercuric iodide on a percentage basis would suggest that ammonium iodide is three times as effective as mercuric iodide (table 1). Actually, however, they are equally active, molecule for molecule. As a matter of convenience, both molar and percentile concentrations are given in the tables.

ing the course of the study, 99.4 per cent were small strongyles with eight intestinal cells, 0.55 per cent *Strongylus vulgaris*, and 0.05 per cent *Strongylus edentatus*. Although no significant difference in results was observed between the different species, these results refer almost entirely to the larvae of the small strongyles.

Most of the organic compounds studied were obtained from Dr. C. S. Marvel, Department of Chemistry, University of Illinois, while the four iodonium compounds were obtained from the Chemical-Biological Coördination Center, National Research Council, Washington, D. C.

RESULTS

In table 1 are given the results obtained with six inorganic iodine compounds. The lowest lethal concentration of potassium iodide, potassium iodate, and potassium metaperiodate was 0.0005 M., while that of ammonium, mercuric, and sodium iodides was 0.00025 M. This difference is probably not significant, since this much variation was observed in replicate tests run with other compounds.

In table 2 are given the results obtained with nine aliphatic organic iodine compounds. All were active. The least effective of the group was diiodomethane disodium sulfonate, whose lowest lethal concentration was 0.0025 M. Iodoform, ethyl iodoacetate, and ethylene iodohydrin (1 test) were effective at 0.001 M. Ethylene iodide (1 test), iodoacetic acid (1 test), *beta*-iodopropionic acid, and ethyl-*beta*-iodopropionate were effective at 0.0005 M. Ethylene iodide (1 test) and ethylene iodohydrin (1 test) were effective at 0.00025 M., the lowest concentration tested at the time. In another test, iodoacetic acid and ethyl-*beta*-iodopropionate were effective in a concentration of 0.0001 M., the lowest concentration tested. Probably the most effective compound in the group was trimethylene iodoacetate, which killed the larvae at a concentration of 0.0001 M. in one test and at least 0.00005 M. in another test.

In table 3 are given the results obtained with iodobenzene and 26 of its derivatives. The lowest lethal concentration of six of these compounds was 0.01 M. That of 19 others was more than 0.01 M. Iodobenzene itself was effective in a concentration of 0.025 M. The most active compounds in this group were 2-nitro-6-methyliodobenzene, whose lowest lethal concentrations were 0.005 and 0.0025 M., respectively, in two tests, and 2-carboethoxyiodobenzene, whose lowest lethal concentration was 0.0025 M. The lowest lethal concentration of 2-carbomethoxyiodobenzene was less than 0.0025 M. in one test and 0.01 M. in another.

In table 4 are given the results obtained with seven heterocyclic iodine compounds. The least effective of this group were vioform (5-chloro-7-iodo-8-hydroxyquinoline) and tetraiodophthalic anhydride. They were ineffective in the highest concentrations used, 0.025 M. and 0.05 M., respectively. Pyridine methiodide and its 2, 4, 6-triethyl derivative, *gamma*-collidine methiodide, were both effective at a concentra-

TABLE 2—Effect of Aliphatic Organic Iodine Compounds on Horse Strongyle Larvae in Manure

Compound	Formula	Lowest lethal concentration*	
		Molar	Per cent
Iodoform	CHI_3	0.00100	0.0400
Ethylene iodide	CH_2ICH_2I	0.00050 <0.00025	0.0140 <0.0070
Ethylene iodohydrin	CH_2ICH_2OH	0.00100 <0.00025	0.0170 <0.0040
Iodoacetic acid	CH_2ICOOH	0.00050 <0.00010	0.0090 <0.0020
Ethyl iodoacetate	$CH_2ICOOC_2H_5$	0.00100	0.0210
Beta-iodopropionic acid	CH_2ICH_2COOH	0.00050	0.0100
Ethyl-*beta*-iodopropionate	$CH_2ICH_2COOC_2H_5$	<0.00010 0.00050	<0.0020 0.0110
Trimethylene iodoacetate	$CH_2ICOOCH_2CH_2CH_2OOCCH_2I$	0.00010 <0.00005	0.0041 <0.0020
Diiodomethane disodium sulfonate	$NaOSO_2CI_2O_2SONa$	0.00250	0.1200

*Each figure is the result of a separate test.

tion of 0.00025 M., although, in one test, the lowest lethal concentration of the trimethyl derivative was 0.001 M. The lowest lethal concentration of piperidine hydroiodide was 0.0005 M.; that of N-N-dimethyl piperidine iodide was 0.00025 M. As mentioned earlier for the inorganic iodine compounds, this difference may not be significant. This view is borne out by the results obtained with 1, 2, 6-trimethyltetrahydroquinoline hydroiodide, whose lowest lethal concentrations were 0.0005 M. and 0.00025 M., respectively, in two tests.

In table 5 are given the results obtained with twelve miscellaneous organic iodine compounds. The three quaternary ammonium compounds, triethyl butyl ammonium iodide, triethyl benzyl ammonium iodide, and phenyl trimethyl ammonium iodide, were about equally effective, their lowest lethal concentrations being 0.0005 M. to 0.00025 M., although, in one test, the lowest lethal concentration of phenyl trimethyl ammonium iodide was at least 0.0001 M. (the lowest concentration tested).

The lowest lethal concentration of triphenyl methyl phosphonium iodide was 0.0005 M., that of tetramethyl arsenic iodide was 0.0005 M. in one test and at least 0.0001 M. in another, and that of trimethyl sulfonium iodide was 0.0001 M.

The least effective iodonium compounds were bis-(p-iodophenyl) iodonium chloride, whose lowest lethal concentration was

TABLE 3—Effect of Iodobenzene Derivatives on Horse Strongyle Larvae in Manure

Derivative	Lowest lethal concentration*	
	Molar	Per cent
Iodobenzene	0.0250	0.510
2-bromo, 4-nitro	>0.0250	>1.000
2-bromo, 4-methyl, 6-nitro	0.0500	0.850
2-bromo, 4-carboethoxy	0.0100	0.355
2, 4, 6-tribromo, 5-methyl	>0.1000	>4.550
2, 4, 6-trichloro	0.0100	0.307
3-nitro, 4-methyl	0.0100	0.263
2-nitro, 6-methyl	0.0050 0.0025	0.131 0.065
3-nitro, 4-methyl, 6-amino hydrochloride	>0.0100	>0.234
2-carboxy	0.0500	1.240
2-carbomethoxy	0.0100 <0.0025	0.262 <0.065
2-carboethoxy	0.0025 0.0025	0.069 0.069
2-methoxy, 5-carboxy	>0.0100	>0.278
2-methoxy, 5-carbomethoxy	>0.0250	>1.000
2-nitro, 6-carboxy	>0.0100	>0.293
2-nitro, 6-carbomethoxy	>0.0100	>0.307
3-carboxy, 4-amino	>0.0250	>1.000
3-carbomethoxy, 4-amino	0.0100	0.277
4-methoxy	0.0100 0.0050	0.234 0.117
2-methoxy, 4-nitro	>0.1000	>2.790
2-methoxy, 5-nitro	>0.0100	>0.279
2-nitro	>0.0100	>0.249
4-acetamido	0.0100	0.261
2-amino, 3-iodo, 4-methyl, 5-nitro	>0.0100	>0.402
2-amino, 3-iodo, 5-carboethoxy	>0.0100	>0.417
2, 3-diiodo, 5-nitro, 6-methyl	>0.0100	>0.515
2, 3-diiodo, 5-carboethoxy	>0.0250	>1.000

*Each figure is the result of a separate test.

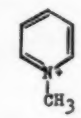
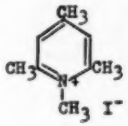
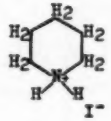
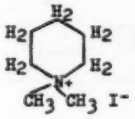
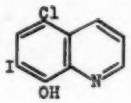
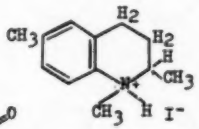
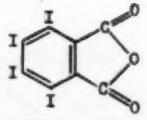
greater than 0.01 M., and bis-(p-chlorophenyl) iodonium chloride, whose lowest lethal concentration was 0.005 M. The two most effective iodonium compounds were diphenyl iodonium iodide and bis-(p-iodophenyl) iodonium iodide, whose lowest lethal concentration was 0.0005 M.

The least effective compounds in this group were 2-iodobiphenyl, whose lowest lethal concentration was 0.01 M., and 1-iodo-2-methyl-4-nitronaphthalene, which was ineffective in the highest concentration tried, 0.01 M.

DISCUSSION

Iodine compounds in general appear to be toxic to strongyle larvae and/or eggs. The results obtained in this study indicated that probably the most important factor determining the degree of toxicity of a compound was the way in which the iodine atom was attached to the molecule. The least effective compounds were those in which the iodine atom was attached directly to a carbon atom of a ring; the most effective organic compounds were those in which the iodine was not attached to a ring car-

TABLE 4—Effect of Heterocyclic Iodine Compounds on Horse Strongyle Larvae in Manure

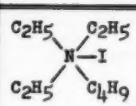
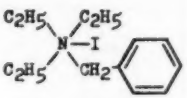
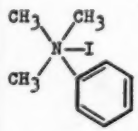
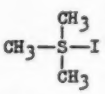
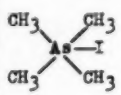
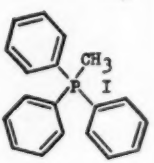
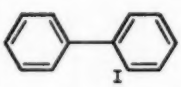
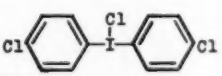
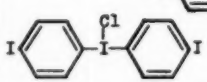
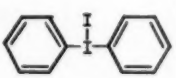
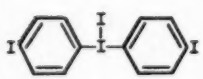
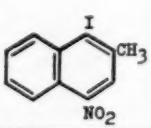
Compound	Structural Formula	Lowest Lethal Concentrations[a]	
		Molar	Per Cent
Pyridine methiodide		0.00025 0.00025	0.005 0.005
Gamma-collidine methiodide		0.001 0.00025	0.032 0.008
Piperidine hydroiodide		0.0005 0.0005	0.010 0.010
N, N-dimethyl piperidine iodide		0.00025 0.00025	0.006 0.006
Vioform (5-chloro-7-iodo-8-hydroxy quinoline)		>0.025	>0.492
1,2,6-trimethyltetrahydroquiniline hydroiodide		0.0005 0.00025	0.019 0.009
Tetraiodophthalic anhydride		>0.05	>3.255

[a] Each figure is the result of a separate test.

bon. Thus, the inorganic and aliphatic compounds were all active, while iodobenzene and its derivatives were all relatively inactive. The pyridine and piperidine compounds, in which the iodine was not attached directly to a ring carbon, were very active. Vioform, a quinoline compound whose iodine atom is attached to a ring carbon, was inactive, while a quinoline hydroiodide derivative was very active.

Increasing the number of iodine atoms attached to ring carbons did not increase the activity of the compounds. Two iodobenzene derivatives containing a second iodine atom and two containing two other iodine atoms were inactive at the highest concentrations studied, as was tetraiodophthalic anhydride. Similarly, all the onium compounds, except the two iodonium compounds in which the only iodine atoms

TABLE 5—Effect of Miscellaneous Organic Iodine Compounds on Horse Strongyle Larvae in Manure

Compound	Structural Formula	Lowest Lethal Concentration*	
		Molar	Per Cent
Triethyl butyl ammonium iodide		0.0005 0.00025	0.014 0.007
Triethyl benzyl ammonium iodide		0.00025	0.008
Phenyl trimethyl ammonium iodide		0.0005 <0.0001	0.013 <0.003
Trimethyl sulfonium iodide		0.0001	0.002
Tetramethyl arsenic iodide		0.0005 <0.0001	0.013 <0.003
Triphenyl methyl phosphonium iodide		0.0005	0.025
2-iodobiphenyl		0.01	0.280
bis-(p-chlorophenyl) iodonium chloride		0.005	0.192
bis-(p-iodophenyl) iodonium chloride		>0.01	>0.568
Diphenyl iodonium iodide		0.0005	0.020
bis-(p-iodophenyl) iodonium iodide		0.0005	0.033
1-iodo-2-methyl-4-nitronaphthalene		>0.01	>0.413

* Each figure is the result of a separate test.

were attached directly to ring carbons, were relatively active. The relatively low toxicity of diiodomethane disodium sulfonate was probably due to steric hindrance.

It is probable that the iodide ion is responsible for the toxic action of these compounds and that its availability determines, in great measure, the degree of activity. Further work is desirable to relate the degree of availability to the degree of activity. In addition, the search should be continued for groups which enhance the activity of the iodine.

The iodate and metaperiodate ions were about as effective as the iodide ion in the inorganic compounds studied.

The results obtained with these iodine compounds suggest that some of them may have practical value as anthelmintics. Further studies must be made, however, to determine whether they are effective *in vivo*.

SUMMARY

The lethal action of 61 organic and inorganic iodine compounds on horse strongyle larvae and/or eggs in feces was studied. Compounds in which the iodine atom was attached directly to a carbon atom in a benzene or other ring were relatively inactive compared with inorganic, aliphatic, onium, and heterocyclic ring compounds in which the iodine was not attached to a ring carbon.

Compounds which were lethal at a concentration of 0.0005 M. or less were ammonium iodide, red mercuric iodide, potassium iodide, sodium iodide, potassium iodate, potassium metaperiodate, ethylene iodide, iodoacetic acid, *beta*-iodopropionic acid, ethyl-*beta*-iodopropionate, trimethylene iodoacetate, pyridine methiodide, *gamma*-collidine methiodide, piperidine hydroiodide, N, N-dimethyl piperidine iodide, 1, 2, 6-trimethyltetrahydroquinoline hydroiodide, triethyl butyl ammonium iodide, triethyl benzyl ammonium iodide, phenyl trimethyl ammonium iodide, trimethyl sulfonium iodide, tetramethyl arsenic iodide, triphenyl methyl phosphonium iodide, diphenyl iodonium iodide, and bis-(p-iodophenyl) iodonium iodide.

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Morphologic Studies of Hog Cholera Virus by Electron Microscopy

REGINALD L. REAGAN; A. L. BRUECKNER, V.M.D.; LEO J. POELMA, D.V.M.

College Park, Maryland

THE MORPHOLOGY of the virus of hog cholera, as revealed by the electron microscope, has been studied by Boynton and his associates.[1] The study to be reported was undertaken in an attempt to obtain electron micrographs to show individual virus particles rather than in an aggregate mass.

EXPERIMENTAL

In preparation of the serum,* 3 pigs, injected with hog cholera virus of proved virulence, were bled aseptically six days after inoculation, at which time typical symptoms of hog cholera had developed. Autopsy revealed lesions usually associated with this virus disease. The blood was defibrinated by shaking, refrigerated for twenty-four hours, and the serum was removed. No preservative was added to the serum before it was shipped in dry ice to our laboratory.

On receipt at this laboratory, this material was thawed and filtered through a type ST size L3 Seitz filter. Of the filtrate, 100.0 cc. was subjected to centrifugation at 44,000 revolutions per minute for three hours in a Spinco ultracentrifuge, with a temperature change of approximately 1 degree. Of the supernate, 3.0 cc. was injected subcutaneously into a normal pig (pig 3, weight 50 lb.) to determine if all virus had been removed by centrifugation.

The sediment was resuspended in 1.0 cc. of 3 per cent saline solution. Of this concentrated material, 0.8 cc. was placed on 50 parlodion film supports, excess fluid was removed with a fine capillary pipette, and the films were dried. The following day, they were shadowed with 40 mg. of chromium[2,3] at a tangent of 1/8. These shadowed screens were examined in an RCA electron microscope, type EMU.

The remaining 0.2 cc. of this resuspended material was diluted to 5.0 cc. with physiologic saline solution. Each of 2, 50-lb. normal pigs (pigs 1 and 2) was injected subcutaneously with 2.5 cc. of the resuspended material. Hog cholera antiserum (70.0 cc.) was injected into pig 2 at the same time.

There was an increase in body temperature in approximately forty-eight hours in all pigs. Symptoms of acute hog cholera developed in pigs 1 and 3. Pig 2 recovered from the increased temperature and returned to normal. Pig 1 was bled and destroyed on the seventh day after inoculation, and pig 3 died during bleeding on the eighth day. Lesions of acute hog cholera were noted at autopsy on each individual.

A serum concentrate was prepared from the blood of pig 1 and electron studies were carried out as above. Pooled blood was obtained from 3 normal pigs and was subjected to the same methods of preparation, centrifugation, and electron microscope examination to observe the extraneous material usually found in normal hog serum.

Figure 1 shows concentrated material from the original serum of pigs injected with a virulent strain of hog cholera virus. Figure 2 shows concentrated material from the serum

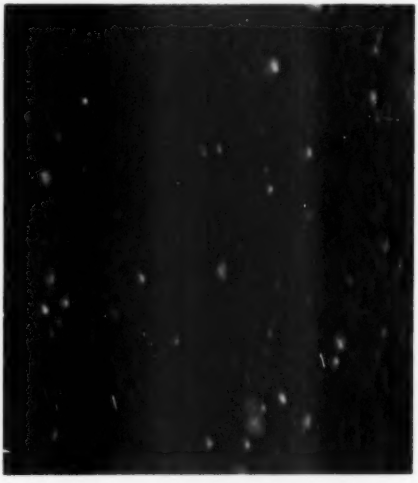

Fig. 1—Electron micrographs of the concentrate of hog cholera serum, shadowed with chromium at tangent 1/8. x 76,800.

From the Live Stock Sanitary Service, Maryland State Board of Agriculture, University of Maryland, College Park.

The authors wish to express appreciation to Dr. Edoardo Sacchi, Miss Dorothy Schenk, and Miss H. Odette Werner for their assistance.

*The infected serum for this work was furnished by Dr. W. T. Munce of the Allied Laboratories, Inc., Sioux City, Iowa.

of a pig injected with the concentrate from the original serum. Both micrographs show virus-like particles, round and varying in size from 22 to 30 mμ by direct measurement. The mean diameter is 27 mμ. Normal hog serum, treated with the same technique of concentration and electron examination, showed none of these round bodies. Boynton and his co-workers[1] prepared electron micrographs of particles presumed to be hog cholera virus which was grown in tissue culture. Direct measurement by Boynton of these particles was 5 mμ in his tissue culture studies.

DISCUSSION

The serum of the pig used for testing the infectivity of the concentrate of the original virus-bearing serum showed virus-like particles similar to those demonstrated in the original serum. The pigs given the concentrate and the supernate were moribund before being slaughtered. The pig, treated simultaneously with the concentrate of the original virus-bearing serum and commercial hog cholera antiserum, showed signs of illness but recovered and remained healthy throughout the remainder of the observation period.

TABLE I

Pig inoculated	Inoculum	Incubation period (days)	Autopsy findings
1	3.0 cc. supernatant	3	Petechial hemorrhages in kidneys, bladder, and epiglottis; hemorrhagic infarcts in spleen; gastroenteritis; ulcerated ileocecal valve; pneumonia.
2	2.5 cc. sediment and 70.0 cc. hog cholera antiserum	2 (recovered by 8th day)	. . . . .
3	2.5 cc. sediment	2	Hemorrhagic lymph nodes, kidneys and bladder; colitis, gastritis, ulcerated ileocecal valve; pneumonia.

The possibility that the virus-like particles demonstrated in the concentrated material are not the true cholera virus can not be discounted in these studies. The fact that the supernate from ultracentrifugation produced the typical disease in a pig indicates that the true virus was not all removed by this process; the fact that the concentrate produced the disease in a pig indicates that the true virus was thrown down in the operation or was attached to the particles thrown down. The absence of another virus capable of producing a fatal disease in the injected pigs was shown by the fact that hog cholera antiserum furnished protection in the pig injected. More extensive trials are indicated.

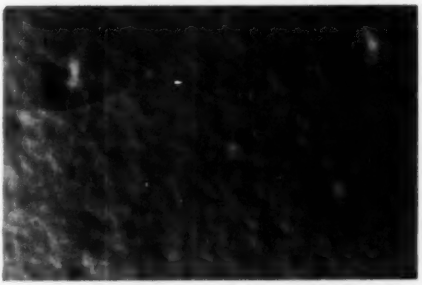

Fig. 2—Electron micrograph of virus-like particles found in concentrate of the serum of a pig infected with concentrate of original hog cholera serum, shadowed with chromium at tangent 1/8. x 112,800.

SUMMARY

Electron micrographs of concentrate of serum of pigs infected with hog cholera show virus-like particles which may be hog cholera virus. These particles are round and have a mean diameter of 27mμ by direct measurement of electron micrographs. These virus-like particles could not be demonstrated in normal pig serum using the same technique of centrifugation and electron examination as used for the infected hog cholera serum.

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The Fertility of Boars Reared in a Restricted Environment

L. E. HANSON, M.S.A., Ph.D.

Lincoln, Nebraska

A SEARCH of the literature reveals little about the nutritional requirements of boars, quantitatively or qualitatively. Neither are there data to support the more or less standard management advice, "feed the boar a well-balanced, nutritious ration and see that he gets plenty of exercise."

Every year, thousands of boars are sold for breeding and some of them never breed.

Fig. 1—Boar 17, series 1, after completion of the breeding season.

Some breed only after treatment. In most cases, a critical evaluation of the various treatments is not possible. The infertility of boars, whether of a temporary or permanent nature, is attributed to numerous causes by different individuals. One of the most frequently expressed opinions is "a lack of exercise." Infertility is the cause of a serious economic loss not only to the breeder who produces the boar but to the purchaser as well, because of a delayed farrowing season and destroyed plans. Clearly, there is a need for specific information about the nutritional requirements of boars for satisfactory reproduction. Information is also needed on management.

In the production of female pigs under restricted conditions for use in other research, 7 boars were saved from two litters farrowed in 1945. In 1947, 6 boars were saved from two litters.

The objective of the studies to be reported was to determine whether boars reared in close confinement would be fertile.

The rearing of the 13 boars and the results of the fertility tests made are reported in two series. The 1945 pigs are included in series 1, and the others in series 2.

SERIES 1

Origin of Boars.—It is an accepted fact that previous nutritional history does influence the results obtained in many experiments. Therefore, the feeding and management of the dams of the boars are reviewed briefly.

In January, 1945, 5 fall-farrowed Duroc-Jersey gilts, which had been kept on concrete floors since the age of 10 weeks, were penned in a barn on concrete floors. They were hand fed a ration composed of ground yellow corn (70%), tankage (9.5%), soybean-oil meal (9.5%), ground fourth-cutting alfalfa (10%), steamed bone meal (0.5%), and salt (0.5%). In addition, approximately 6 Gm. of irradiated dry yeast were added to each 100 lb. of mixed feed. In April, 1945, linseed meal (2%) was added to make the ration more laxative; the corn was increased and the tankage and soybean meal decreased in order to reduce the protein content of the diet. The average initial weight of the gilts was 78.6 lb. and, at the time of breeding in the latter part of April, it varied from 178 to 237 lb. each. Four of the 5 gilts conceived during the first estrus in which they were bred. The fifth was bred twice each estrous period for four consecutive periods, but did not conceive.

At farrowing time (Aug. 11 to Aug. 22, 1945), the pregnant gilts weighed from 421 to 510 lb. The first to farrow had 9 live pigs. She overlaid 3, but raised 4 boars (No. 6, 7, 8, and 9) and 2 gilts. Boar 6 was blind in both eyes. The second gilt farrowed 10 live pigs and 1 dead pig. Of the live pigs, 1 had no left eye and a deformed pelvis. Initiation of lactation was delayed and most of the litter starved, including the defective pig. Only 3 pigs survived, boars 17, 18, and 19.

The third litter included only 6 live pigs. Their dam refused to accept them and all

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Acknowledgment is made of the assistance of Merle Brinegar, Donald Warner, Robert J. Meade, and O. L. Shaffer, who fed and cared for the animals and made the matings.

At present, Dr. Hanson is in the Division of Animal Husbandry, University of Minnesota, St. Paul.

starved. One of these had a severe congenital contracture of the tendons of the hind limbs. The fourth gilt, a litter mate of the first, farrowed 7 live and 2 dead pigs. Five of the live ones were deformed. The deformities varied from too many or too few toes on the hind feet to 1 pig which lacked one hind limb and part of the other hind limb. None of the pigs in this litter survived. All of the live pigs farrowed in the 4 litters were vigorous at birth, even those that were deformed.

Whether the deformities were of genetic or nutritional origin is not known. The sire of the deformed pigs was bred in the Nebraska Agricultural Experiment Station herd. However, he was related to the gilts with which he was mated only through the female side of the pedigree, and the inbreeding of the deformed pigs was approximately 5.0 per cent. The death of the boar before the following breeding season prevented the repetition of the matings that produced the deformities. Work at Texas[1] and Wisconsin[2,3] indicates that such defects can have a nutritional origin.

During the suckling period, the gilts were fed rations of the same ingredients as before, but the protein content was increased slightly by reducing the corn and increasing the proportion of tankage and soybean-oil meal. Also, the linseed meal was increased to 3 per cent as the gilts were becoming slightly constipated.

During the growth period, the 5 gilts were confined together in one 10- by 10-ft. pen. During gestation, they were penned 2 to each 10- by 10-ft. pen and were penned separately only a few days before farrowing. The only exercise they received was what they took in these small pens and a 70-ft. walk to the scales every fourteen days.

Experimental Procedure.—Four boars and 2 gilts from the first little (farrowed Aug. 11, 1945) and the 3 boars from the second litter (farrowed Aug. 14) were weaned Oct. 6, 1945, at an average weight of 28 lb. (range 24 to 31 lb.). These pigs were hand fed twice daily (*ad lib.*) as one lot until March 23, 1946. On that date, the average weight of the 9 head was 244 lb. They had made an average daily gain of 1.29 lb. and required 4.0 lb. of feed per pound of gain. The rations fed were composed of varied amounts of ground yellow corn, tankage, and soybean meal, and included alfalfa meal (10%), distillers' solubles (5%), linseed meal (2%), steamed bone meal (0.5%), and salt

TABLE 1—Breeding Record of 7 Duroc-Jersey Boars Reared Under Restricted Conditions and the Farrowing Record of 32 Females to Which They Were Mated (1945-1946)

Gilt (No.*)	Bred to boar (No.)	Date bred	Date farrowed	No. live pigs	No. dead pigs	Remarks
D591-H	6	April 28	Aug. 18	9	2	
Tried sow	6	May 4, 5	Aug. 25	6	0	
D545-H	6	May 10, 11	Aug. 29	11	0	
H560-T	6	May 12, 13	Sept. 4	5	1	
D547-T	6	May 15, 16	Sept. 8	11	3	
D571-T	6	June 4, 5	Sow died before farrowing			
D555-H	7	April 28	Aug. 19	9	0	
D172	7	May 6, 7, 8	Did not conceive			
D576-T	7	May 11	Sept. 3	8	0	
LD561-W	7	May 13, 14	Sept. 2	11	0	
D373	7	May 30	Sept. 20	11	1	
D415	7	June 5, 6	Sept. 27	9	0	
LD552-W	8	April 30, May 1	Sept. 9	5	0	
D583-L	8	May 6, 7	Aug. 28	9	0	1 blind pig
D563-H	8	May 10, 11	Aug. 31	12	0	
D572-T	8	May 12, 13	Sow died before farrowing			
LD553-W	8	May 17, 18, 19	Sept. 5	8	0	
D565-H	8	May 28	Sept. 18	6	0	
D172	8	May 28, 29	Sold on the market—apparently pregnant			
D551-H	9	April 28, 29	Aug. 17	6	0	
D538-T	9	May 1, 2	Aug. 24	8	3	
LH577-W	9	May 8, 9	Aug. 29	8	0	
D558-H	9	May 11, 12, 13	Sept. 1	11	0	
D573-L	9	May 16, 17	Sept. 5	8	0	
D593-T	9	June 1, 2, 3	Sept. 15	13	1	107-day gestation
D550-T	17	May 3, 4	Aug. 27	5	2	
D585-H	17	May 7, 8	Aug. 28	9	0	1 blind pig
LH554-W	17	May 11, 12	Aug. 30	9	0	
D574-Wa	17	May 13, 14	Sept. 4	13	0	
LH552-W	17	May 19, 20, 21	Sept. 9	5	0	
D4	18	April 28	Aug. 18	7	0	
D410	18	April 30	Aug. 22	9	1	
D370	18	May 1	Aug. 24	12	0	
D373	18	May 9, 10	Did not conceive			
D415	18	May 12	Did not conceive			
LD553-W	19	April 28, 29	Did not conceive			
D565-H	19	May 6, 7	Did not conceive			
D401	19	May 9, 10	Aug. 30	12	0	
D593-T	19	May 11, 12	Did not conceive			
D571-T	19	May 13, 14	Did not conceive			

*D = Duroc-Jersey; H = Hampshire; LH and LD = Crossbred. Initials following gilts' numbers refer to the farmers who purchased them.

(0.5%). The corn, tankage, and soybean meal content of the ration was varied during the feeding period to obtain the desired protein level. In addition, approximately 6 Gm. of irradiated dry yeast were added to each 100 lb. of mixed feed.

The 4 boars (6, 7, 8, and 9) from the first litter began to show definite sex interest when approximately 5 months of age. By March 23, 1946, 1 was losing weight and another was making practically no gain. The 3 boars (17, 18, and 19) from the second litter were somewhat slower in reaching sexual maturity. All of the boars were continued on the same ration after the end of the feeding period mentioned previously and through the breeding period. The latter extended from April 28 to June 6, 1946, inclusive.

Twenty-five fall-farrowed gilts were used in testing the boars. They were farrowed on pasture, under normal conditions, but were placed in concrete-floored pens at 9 to 12 weeks of age, where they were fed until breeding was completed. Three to four weeks before breeding began, they were turned out daily on a brome grass-alfalfa pasture. Six additional fall-farrowed gilts were used. These were reared in the barn under the same conditions as the boars. One of these was a litter mate to boars 6, 7, 8, and 9. The other 5 were farrowed on pasture, but were placed in the barn, at an average weight of 52.4 lb., on Dec. 29, 1945. In addition to these, 1 tried sow was used. She had been raised and managed under normal conditions.

Twenty-four of the gilts were sold to five Nebraska farmers after it became apparent that the gilts were pregnant. One gilt was marketed because of blind teats. She appeared to be pregnant. The 6 gilts raised in the barn and the tried sow were retained.

RESULTS

It is apparent from the results presented in table 1 that all of the boars were fertile. However, the record of boar 19 was poor. The reason or reasons for this were not apparent. On July 8, 1946, the boars were castrated and the testicles examined by the college veterinarian. He reported, "The examination of the spermatic fluid from the testicles of each of the seven boars submitted to this laboratory showed that there was an apparently normal number, morphology, and motility of spermatozoa in all of these animals."

It will be noted that 1 blind pig was sired by boar 8. The latter was a litter mate of boar 6, the blind pig in the first litter. Each farm was visited after the pigs were farrowed, and a careful inspection of the litters was made. The blind pig sired by boar 8 had no left eye and the right eye was sufficiently cloudy to prevent sight. The blind pig sired by boar 17 had no eyes, but he seemed normal in other respects and was thriving when inspected about ten days after birth. The litter from which boars 17, 18, and 19 came contained 1 pig which had one eye missing.

Two of the gilts sold to Mr. T. died before farrowing. The remaining 6 farrowed 10 dead pigs. The 16 gilts sold to four other farmers farrowed only 2 dead pigs, and the 6 gilts and sow that were retained farrowed only 2 dead pigs. Mr. T. had delayed vaccinating his spring pigs for cholera until a few weeks after he obtained the bred gilts from the Nebraska Agricultural Experiment Station. It was believed that the sows contracted cholera, although no postmorten inspection was made. All of the gilts used in this test were vaccinated for cholera by the simultaneous method when they were from 4 to 6 weeks old.

SERIES 2

Origin of Boars.—The 2 gilts that raised the boars reported in series 1 were rebred to a different boar for 1946 spring litters. No defective pigs were produced. Several gilts from these litters were raised on the same ration and in the same restricted environment as their dams. These gilts were bred to a boar produced in the Station herd. Four boars (87, 88, 89, and 80X) from one litter were farrowed April 14, 1947. They were grandsons of the dam of boars 6, 7, 8, and 9 reported in series 1. Two boars (101 and 102) from another litter were farrowed May 8, 1947. They were grandsons of the dam of boars 17, 18, and 19 reported in series 1. Thus, these boars represented the third consecutive generation raised in confinement on concrete floors.

Experimental Procedure.—The 6 boars were self fed as one lot in a 10- by 20-ft. pen, for 105 days after weaning. They made an average daily gain of 1.55 lb. and required 3.6 lb. of feed per pound of gain. Their ration was the same as that fed to the boars in series 1. They were continued on the same ration, hand fed twice daily throughout the breeding period which extended from Oct. 12 to Dec. 27, 1947, inclusive. The boars began to show sex interest at 5 months of age and were used for breeding at 6 to 8 months.

Thirty purebred Hampshire and Duroc-Jersey gilts were used for testing the boars. The gilts were farrowed on pasture, or had access to

pasture before weaning. With a few exceptions, all were raised in concrete-floored pens outdoors. All of them were fed good rations. Most of them had no access to pasture or soil from weaning until after they were bred.

Most of the gilts were sold to local farmers, from two to sixty days after breeding. A few were slaughtered. The boars were sold to other farmers and breeding reports were obtained from them.

RESULTS

The results of the matings are presented in table 2. It is apparent that all of the boars were fertile. The conception rate was at least as high as is usually obtained in the Nebraska Agricultural Experiment Station herd, handled under normal conditions. The farrowing reports were verified by an inspection visit to each farm.

Since the number of gilts available for test mating was limited, the boars were sold to farmers for further testing. The farmers managed the boars in different ways, but none restricted the pigs as was done at the Station.

The following breeding reports were submitted by the farmers:

Boar 87 was sold to H. E. T. on Jan. 10, 1948. The boar was mated with 9 gilts, and they farrowed a total of 72 pigs.

Boar 88 was sold to H. L. on Dec. 15, 1947. Of 15 gilts bred to this boar, 1 did not conceive and 2 were sold to neighbors. The other 12 farrowed a total of 111 pigs.

Boar 89 was sold to L. P. on Jan. 12, 1948. He was mated with 4 gilts and they farrowed a total of 42 pigs.

Boar 80X was sold to R. H. on Jan. 12, 1948. He was mated with 10 gilts. R. H. kept 7 of these and they farrowed a total of 51 pigs.

Boar 101 was sold to L. S. on Dec. 3, 1947. L. S. bred 12 of his gilts and 3 of his neighbor's gilts to this boar. The 15 gilts farrowed a total of 146 pigs. Another farmer purchased the boar from L. S. for use in breeding for 1948 fall litters.

Boar 102 was sold to F. T. on Jan. 14, 1948. He was mated with 6 gilts and they farrowed a total of 54 pigs.

SUMMARY

Thirteen Duroc-Jersey boars were reared

TABLE 2—Breeding Record of 6 Duroc-Jersey Boars Reared Under Restricted Conditions and the Farrowing Record of 30 Females to Which They Were Mated (1947-1948)

Gilt (No.*)	Bred to boar (No.)	Date bred	Date farrowed	No. live pigs	No. dead pigs	Remarks
H102-P	87	Dec. 19	April 10	12	0	
H91-P	87	Dec. 24	....	..	9	Sow died before farrowing, uterus contained 9 pigs
H40-P	87	Dec. 26, 27	April 17	12	0	
H142-P	88	Dec. 9	April 3	11	1	
H172-H	88	Dec. 10	....	..	..	Did not conceive
H103-H	88	Dec. 12	April 3	7	0	
D83	89	Nov. 22	....	..	9	Slaughtered Jan. 15. Uterus contained 9 pigs
H140-P	89	Nov. 26	March 17	7	1	
H90-P	89	Dec. 5	March 28	12	0	
D61-P	89	Dec. 6	March 29	12	0	
H141-L	89	Dec. 8	March 31	10	1	
H41-P	89	Dec. 25	April 16	9	0	
H70-K	80X	Nov. 10	March 1	7	3	
D123-P	80X	Nov. 20	March 13	8	4	
H112-L	80X	Dec. 6	March 29	8	0	
H104-L	80X	Dec. 9	....	..	..	Did not conceive
H113-P	80X	Dec. 19	April 11	10	0	
H92-P	80X	Dec. 24	April 18	11	0	
H140	101	Oct. 18	....	..	..	Did not conceive
D142	101	Nov. 6	....	..	12	Slaughtered Dec. 10. Uterus contained 12 pigs
H175	101	Nov. 7	....	..	?	Slaughtered Dec. 17. Pregnant; no count made of embryos
D120-P	101	Nov. 10	March 4	9	0	
D44-P	101	Nov. 27	March 18	10	0	
D45-K	102	Nov. 9, 10	March 3	9	3	
H151	102	Nov. 11	....	..	..	Slaughtered Dec. 17. Not pregnant
D191-P	102	Nov. 12	March 4	7	2	
H42-P	102	Nov. 20	March 13	11	0	
D47-P	102	Nov. 23	March 17	9	0	
H130-P	102	Dec. 16	April 8	11	0	
H94-P	102	Dec. 26, 27	April 19	12	0	

*H = Hampshire; D = Duroc-Jersey. Initials following gilts' numbers refer to farmers who purchased them.

in a barn on concrete floors with very little room for exercise. They were farrowed by gilts raised under similarly restricted conditions. Seven of the boars were from the second generation raised in the barn. Six were from the third generation likewise raised in confinement. The boars grew well on a diet composed of varied amounts of ground yellow corn, tankage, and soybean-oil meal, and including alfalfa meal (10%), distillers' solubles (5%), linseed meal (2%), steamed bone meal (0.5%), and salt (0.5%), to which was added approximately 6 Gm. of irradiated dry yeast per 100 lb. of the mixture.

The 7 second generation boars began to exhibit sex interest at approximately 5 months of age and were used for breeding at 8½ to 9 months. Thirty-one gilts and 1 tried sow were bred and all apparently conceived. Two gilts died and 1 was marketed before farrowing. The remaining 28 gilts and 1 sow farrowed a total of 274 pigs.

The 6 third generation boars began to exhibit sex interest at about 5 months of age and were used for breeding at 6 to 8 months. They were mated with 30 gilts from the Nebraska Agricultural Experiment Station herd. Four of these did not conceive; 1 conceived and was slaughtered without further record. The remaining 25 gilts farrowed, or were slaughtered and their uteri examined, and a total of 259 pigs were counted.

Six third generation boars later were sold to six farmers who mated them with 59 gilts. Fifty-three of these, on which records were available, farrowed a total of 476 pigs.

A "lot of exercise," direct sunlight, and pasture are not essentials in the production of fertile boars.

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Laminectomy in the Dog

RICHARD W. REDDING, D.V.M., M.Sc.

Davis, California

VETERINARIANS in the United States have recognized paraplegia as a disease entity in the dog for many years. However, until recently, one of its major causes remained undetermined. Riser,[10] after an extended study, stated that protrusion of one or more of the intervertebral discs into the spinal canal is a major cause of paraplegia. Intervertebral disc protrusion* causes compression of the spinal cord. This results in pain and paralysis, the degree depending upon the amount of compression. The purpose of this experiment was to determine the effects of a surgical operation which may be used in an attempt to alleviate this condition.

REVIEW OF RELATED LITERATURE

Feldman[6] reported a case in which a so-called chondroma was found protruding into the spinal canal of a young Dachshund. Paraplegia had developed suddenly.

According to Brook,[5] enlargement of the intervertebral disc was recognized on the European continent as early as 1913. This led to paralysis of the hind legs, with incontinence of urine and feces.

Brumley[4] described two pathologic conditions which he considered to be causes of paraplegia: (a) degeneration of the intervertebral disc, in which there was distortion and enlargement, causing compression of the cord; and (b) ossification of the intervertebral disc, with the resulting enlargement projecting into the spinal canal directly compressing the cord.

Schlotthauer[12] observed in a dog a protruding intervertebral disc which caused paralysis of the hind legs and the urinary and anal sphincters.

A review of related literature[1,2,5,7,9,11] in human medicine reveals that protruding intervertebral discs occur in man. However, in man, the lesion usually occurs in the lumbar region caudal to the termination of the cord proper. Thus, the protruding disc causes pressure on one or more of the nerve roots, not on the spinal cord itself. The result is pain and partial paralysis, not complete paralysis, as occurs in the dog. However, in 1 case reported by Orley,[9] the result was complete loss of power in the lower limbs, with urinary and fecal incontinence. This case presented symptoms remarkably similar to those seen in dogs.

IMPORTANCE OF THE PROBLEM

A surgical operation, laminectomy and removal of the offending disc, has afforded relief in human surgery.[1,7,9,13] A review of veterinary literature fails to reveal a surgical approach to this problem in the dog. In 1946, Riser[10] stated, "Surgery is successfully performed in man in many cases. It remains to be seen to what extent surgery will be useful in the dog." The approach to this problem in human surgery could possibly be adapted to the dog. However, since the vertebral column of the dog differs in size and shape, it is necessary to determine the effect of a laminectomy on the spinal cord and its surrounding structures (bone, nerve roots, and surrounding soft tissue), before the operation is attempted on pathologic cases.

EXPERIMENTAL PROCEDURE

Surgical Anatomy.—An understanding of the anatomy involved in this surgical operation is helpful. Therefore, the following anatomic descriptions are presented.

The skin of the dog is thickest over the dorsum of the back, especially over the lumbar region. The subcutaneous tissue is not remarkable. The cutaneous trunci muscle, which has a fascial extension forming a broad band over the lumbar region, is found just ventral to the subcutaneous tissue. It attaches to the supraspinous ligament. The superficial layer of the lumbodorsal fascia forms a thick sheath over the lumbar region. It also attaches to the supraspinous ligament. The aponeurosis of the longissimus dorsi closely invests that muscle and attaches to the supraspinous ligament and the spinous processes of the lumbar vertebrae. The longissimus dorsi and the multifidus dorsi muscles lie in the angle formed by the lateral and dorsal processes of the lumbar vertebrae (fig. 1). The interspinales muscles lie between the adjacent spinous

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This article is a condensation of a thesis presented in partial fulfillment of the requirements for the degree of master of science. The study was undertaken in the Department of Surgery, College of Veterinary Medicine, Ohio State University, Columbus, under the direction of Dr. W. F. Guard.

At present, Dr. Redding is at the School of Veterinary Medicine, University of California, Davis.

*Also referred to as: herniated disc, prolapsed disc, slipped disc, ruptured disc, and fractured disc.

processes of the lumbar vertebrae. The ligamentum flavum is very small in the dog.

A lumbar vertebra consists of a body, arch (the major portion of which is called the lamina), and two transverse and one spinous process. There are four facets which join the two adjacent vertebrae.

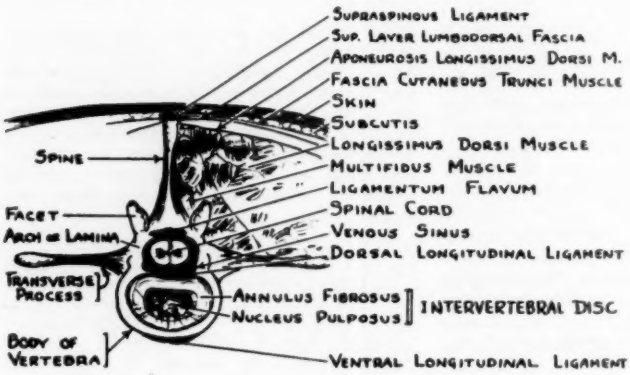

Fig. 1—Cross section of lumbar region.

The vertebrae are held in apposition by the annulus fibrosus, dorsal and ventral longitudinal ligaments, ligamentum flavum, and supraspinous ligament. The ventral longitudinal ligament widens and fuses with the annulus fibrosus, from which it is macroscopically indistinguishable.

Special Surgical Instruments.—The following surgical instruments, used by the author, greatly facilitated the surgical procedure:

Volkman retractor, four prong, sharp.
Lucae bayonet forceps, 5½ in.
Noyes dressing forceps, alligator jaws.
Graham suction tube, 7½ in.
Penberthy aspirator.
Galt trephines.
Periosteal elevator.
Schede bone curette, small.
Luer bone rongeur.
Plain flexible probe with longitudinally flattened end.

In addition to the special instruments, a general operating kit was used.

Preoperative Preparation.—Anesthesia.—Intravenous pentobarbital sodium was administered until deep surgical anesthesia ensued.

Position on the Operating Table.—Ventral recumbent position with the pelvic limbs adducted and pectoral limbs extended was found most satisfactory.

Operative Site.—The operative site was the left lumbar region, extending from the last rib to the wing of the ilium.

Preparation of Operative Site.—The operative site was clipped, shaved, and twice scrubbed with detergent soap and alcohol. Tincture of metaphen was applied and allowed to dry. The area was draped with surgical shrouds.

Operative Procedure.—A review of the radiographs filed in the Veterinary Clinic, Ohio State University, Columbus, reveals that the majority of intervertebral disc lesions have occurred in the lumbar region. Riser's study[10] also indicates this. Therefore, this surgical procedure has been limited to the lumbar region, not only because it is the most common site of pathology, but also to delimit the problem.

The operation was performed on 10 normal dogs. Radiographs were taken immediately before and after the operation. Three additional radiographs of each animal were taken at monthly intervals.

The skin incision was made ½ in. lateral and parallel to the spinous processes of the lumbar vertebrae, beginning approximately 1 in. posterior to the last rib and extending to within 1 in. of the iliac wing. The subcutaneous tissue was incised the length of the skin incision and reflected with the skin. Hemostasis, using mosquito forceps and gauze pressure, was necessary at this point to prevent hemorrhage into the operative area. It was not necessary to ligate the subcutaneous blood vessels. The fascial extension of the cutaneous trunci muscle was incised the length of the incision and reflected. The superficial layer of the lumbodorsal fascia was incised to within ½ in. of the wound commissures. The aponeurosis of the longissimus dorsi muscle was incised to within ½ in. of the wound commissures. A Volkman retractor was hooked into the belly of the muscle and tension applied laterally. Using a periosteal elevator, blunt dissection of the muscle from the spinous processes and articular facets of three adjacent

vertebrae was performed. It was found that traction applied to the muscle would tense the tendinous attachments, so that the belly of a scalpel could be pressed against these attachments, cutting only the tendon and not the adjacent blood vessels. The Graham suction tube proved very useful in removing excess blood throughout blunt dissection. Pressure packs of gauze were applied to the wound and allowed to remain for one to three minutes. This was found absolutely necessary, as too much blood in the field obscured vision, making further surgery impossible. The central one of the three articular facets was freed of all adhering tissue. A trephine with a diameter just large enough to fit over the exposed facet (½, ⅝, or ¾ in.) was positioned. A rotary motion was used in the bone-cutting process. After every five or six turns, the process was interrupted by a mediolateral motion, to determine the depth of cut. When the cut was deep enough, the facet could be seen and felt to move with the trephine. Extreme caution was necessary from this point, since too deep a cut would sever the spinal cord, or puncture the venous sinus, or both. The facet being loosened was grasped with a bone rongeur and extracted. Occasionally, a small shelf of bone would remain. This was removed with a bone curette, being careful to chip outward only. Hemorrhage from the cut bone surface was frequently noted. This was controlled with gauze compression. The epidural fat was removed with meticulous care to prevent damage to the spinal cord, venous sinus, and nerve roots (fig. 2). Noyes dressing forceps and Lucae bayonet forceps were useful in this maneuver. The handle of a flexible probe was fashioned for use as a spinal cord retractor. Elevation of the cord dorsally brought into view the annulus fibrosus of the disc (fig. 2 and 3).

Closure of the wound was comparatively simple. A circular piece of gelfoam (Upjohn), cut to fit the trephine hole snugly, was inserted. The gelfoam acted as a hemostatic agent and cushioned the spinal cord against the overlying tissue. The muscles were returned to their respective positions. All three fascial layers were brought into apposition with a continuous catgut* suture. The skin incision was closed either with simple interrupted silk sutures or with subcuticular catgut sutures. Staple tension sutures were used for tissue relaxation in some cases.

The surgical dressing was composed of a gauze pack applied to the wound and held in place with adhesive tape around the entire body of the animal.

Postoperative care consisted of confinement in a cage for three days, after which the animal was exercised daily.

A laminectomy was performed on each of 10 normal dogs. The operative site was the left side of the lumbar region, either between vertebrae L-2 and L-3, or L-3 and L-4, or L-4 and L-5.

SURGERY AND POSTOPERATIVE OBSERVATIONS

Dog 1.—In attempting to expose the annulus fibrosus, the spinal cord was manipu-

*No. 000 medium chromic.

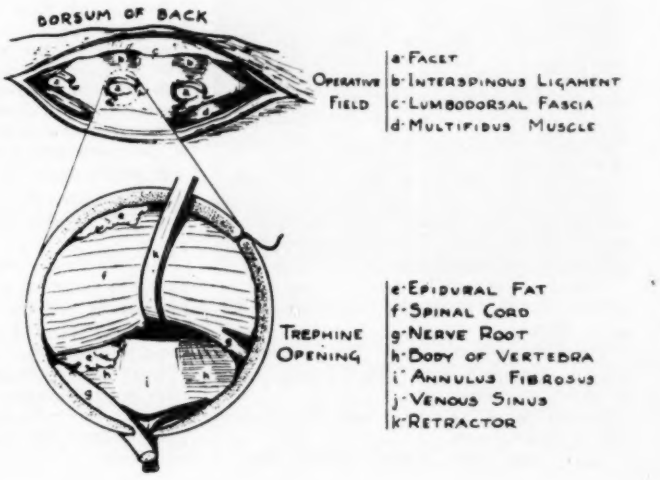

Fig. 2—Operative field and trephine opening.

lated considerably, and the dorsal nerve root was traumatized. Of the 10 dogs operated on, this was the only one in which gelfoam was not used.

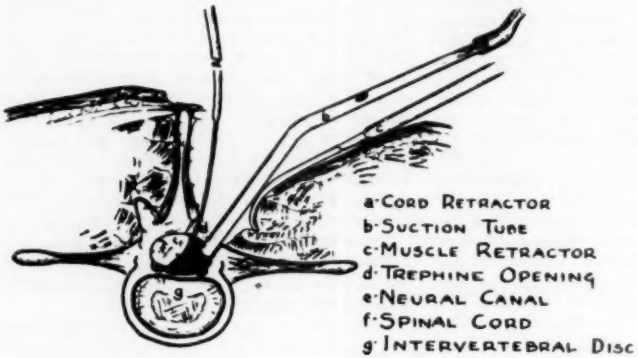

Fig. 3—Surgical exposure and instrumentation.

First Postoperative Day.—The animal evidenced signs of pain and was unable to stand. However, he assumed a rigidly upright sitting position.

Second Day.—The animal showed less pain but was still unable to stand.

Third Day.—The dog was able to rise almost to a standing position. He did not attempt to walk.

Fourth Day.—The animal was able to stand with difficulty. Attempts to walk resulted in failure.

Fifth Day.—The animal was able to stand with less difficulty than on the previous day. Attempts at walking were somewhat more successful.

Sixth Day.—The animal was able to stand and walk with little difficulty. He seemed much improved over the fifth day. The left pelvic limb appeared to have less muscular control than the right.

Seventh to Thirteenth Day.—The animal improved rapidly during this period. On the thirteenth day, he walked normally. From the thirteenth day until autopsy, there was no change in his condition.

Dog 2.—Very little manipulation of the spinal cord was necessary to expose the annulus fibrosus.

First Postoperative Day.—The animal was able to stand with very little difficulty. Little pain was evidenced.

Second Day.—The animal was able to stand and walk with ease.

Third Day.—The animal jumped from a second tier cage to the floor with no resultant pain.

Fourth Day.—The animal appeared normal and continued to be the same until autopsy.

Dog 3.—The venous sinus was accidentally lacerated, which resulted in profuse hemorrhage. Continuation of the operation was impossible due to the excessive blood in the operative area. The opening was packed with gelfoam and the wound closed.

First Postoperative Day.—The animal was unable to rise and not inclined to move.

Second Day.—He made unsuccessful attempts to stand.

Third Day.—The animal was able to rise and stand for a short period.

Fourth and Fifth Day.—There was no discernible change.

Sixth Day.—The animal was able to walk with considerable difficulty.

Seventh to Ninetieth Day.—The animal did not regain ability to walk normally. There was posterior weakness and atrophy of the muscles of the pelvis and pelvic limbs.

Dog 4.—There was excellent exposure of the spinal cord and annulus fibrosus in this dog. The amount of hemorrhage was negligible.

First Postoperative Day.—The animal was unable to stand but appeared to be comfortable in a sitting position.

Second Day.—The animal was able to stand for a short period.

Third Day.—The animal was able to walk with some difficulty.

Fourth Day.—No observation.

Fifth Day.—The animal was able to walk with ease.

Sixth Day.—The dog was apparently normal and continued to be the same until autopsy.

Dog 5.—There was considerable hemorrhage during the operation.

First Postoperative Day.—The animal was unable or unwilling to rise to a sitting position. However, she crawled about on the cage floor.

Second Day.—The animal was unable to stand.

Third Day.—The animal was able to stand and walk with some difficulty.

Fourth Day.—No observation.

Fifth Day.—The animal was able to walk, however, marked posterior weakness was observed.

Sixth Day.—There was improvement over the previous day.

Seventh Day.—The animal was able to walk and run. From the seventh day until autopsy, there was no change in her condition.

Dog 6.—There was very little hemorrhage during the operation.

First Postoperative Day.—The animal was able to rise and stand with little pain.

Second Day.—The animal was able to walk and run without difficulty.

Third and Fourth Day.—No observation.

Fifth Day.—The animal appeared normal and continued to be the same until autopsy.

Dog 7.—There was some hemorrhage during the operation.

First Postoperative Day.—The animal was able to stand with difficulty.

Second Day.—The animal was able to walk with difficulty.

Third Day.—The animal was able to walk with less difficulty than on the previous day.

Fourth Day.—There was no discernible change.

Fifth Day.—The animal walked without difficulty. However, there was generalized weakness.

Sixth and Seventh Day.—The general condition of the animal improved.

Eighth Day.—The animal was apparently normal and continued to be until autopsy.

Dog 8.—The amount of hemorrhage was negligible.

First Postoperative Day.—The animal was able to walk with difficulty.

Second Day.—There was slight improvement over the previous day.

Third Day.—There was no discernible change.

Fourth Day.—The animal was apparently normal and remained the same until autopsy.

Dog 9.—The vertebrae of this animal were very soft and bleeding was excessive. (The animal was immature.)

First Postoperative Day.—The animal was able to stand with difficulty.

Second Day.—The animal was able to walk with difficulty.

Third and Fourth Day.—The animal continued to improve.

Fifth Day.—The animal was apparently normal and continued to be so until autopsy.

Dog 10.—Hemorrhage was not excessive during the operation.

First Postoperative Day.—The animal was able to stand with difficulty.

Second Day.—The animal was able to walk with difficulty.

Third Day.—The animal was able to walk with ease.

Fourth Day.—The animal was apparently normal and continued to be the same until autopsy.

Radiography

Radiographs were made prior to surgery to determine any defects the animal may have had and to provide a preoperative radiograph for comparison. Radiographs were made immediately following surgery and, in order to observe changes during healing, every thirty days thereafter.

Interpretation of the radiographs of all 10 dogs suggested that only fibrous tissue filled the surgical defect. In no case was calcification or ossification of the defect shown radiographically. Figure 4 illustrates the gradually increasing density in the area of the defect.

Necropsy

All 10 dogs were destroyed approximately ninety days following surgery. In each dog, the trephine hole was filled with fibrous tissue in the regressive stage. In dog 3, there was an extensive adhesion between the dura mater and the scar tissue filling the surgical defect. The adhesion was approximately 2.5 cm. long. The spinal cord was constricted and drawn toward the scar. In dog 5, there was a small

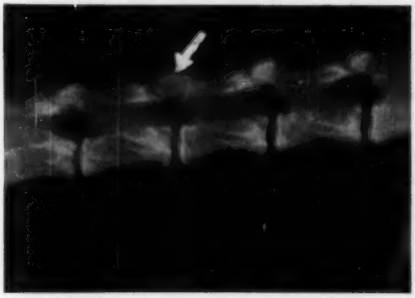

Fig. 4—Postoperative radiograph, fifty-nine days.

adhesion, approximately 1 cm. long, between the dura mater and the scar. The cord was not drawn over by the cicatrix.

It was interesting to note that the twenty-day chromic catgut suture in the lumbodorsal fascia was intact and retained some of its strength at the end of the ninety-day healing period.

SUMMARY AND CONCLUSIONS

Veterinarians have recognized paraplegia in the dog for many years. However, until recently, one of its major causes, intervertebral disc protrusion, remained undetected. The purpose of this experiment was to determine the effects on the dog of a laminectomy, similar to that used in human surgery for correction of protruding intervertebral discs.

The anatomy of the surgical area is reviewed in detail. The surgical instruments used in the experiment are listed, and their use in the operation is described. A description of the operative procedure follows.

An incision was made through the skin and lumbodorsal fascia. The muscles were retracted, exposing the facets of three adjacent vertebrae. A trephine opening was made into the spinal canal, using the central of the three facets as a guide. The spinal cord was elevated to expose the annulus fibrosus. It appeared that the amount of exposure afforded by the trephine opening would be adequate to permit operation on a protruding intervertebral disc. The surgical wound was closed and the animal observed for ninety days.

All 10 animals used in the experiment were paralyzed to some degree on the first postoperative day. The length of time required for the animals to regain ability to walk varied from one to six days. One dog, in which the venous sinus was lacerated during the operation, had not recovered after ninety days. Apparently, there was a relationship between the amount of hemorrhage in the operative area and the recovery time—the more hemorrhage, the longer recovery time.

Radiographs made at thirty-day intervals failed to show ossification or calcification of the surgical defect in the vertebrae.

Autopsy of the animals at the end of the ninety days revealed that the surgical defect was filled with fibrous tissue in the regressive stage of healing. In the animal that remained partially paralyzed, the dura mater had adhered to the fibrous tissue and had constricted and displaced the spinal cord laterally, this also being the animal in which the venous sinus was lacerated during the operation.

Laminectomies performed on 10 normal dogs were without serious sequelae in 9. Results indicate that the operation might offer a satisfactory approach in the surgical treatment of protruding intervertebral discs.

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Newcastle Disease and Infectious Bronchitis Neutralizing Antibody Indexes of Normal Chicken Serum

CHAS. H. CUNNINGHAM, M.S., D.V.M.

East Lansing, Michigan

THE INCREASING importance of Newcastle disease and infectious bronchitis of chickens has emphasized the necessity for differential diagnoses.

LITERATURE

Differentiation of these diseases based on clinical symptoms and lesions and the characteristic effect of the viruses in embryonating chicken eggs have been reported.[1,2,3,5,7,8,9,10,11,15,20,21] Newcastle disease virus (NDV) and infectious bronchitis virus (IBV) are capable of stimulating the production of antibodies which can be detected by *in vitro* serum-neutralization tests.[2,3,12,13,14,16,17,18,20,21] In many instances, such serologic evidence is the only criterion available for diagnosis of prior infection. If preinfection antibody levels are not known, the quantitative significance of post-infection antibody levels must be evaluated for diagnostic purposes by comparison to values considered to be representative of the normal antibody level of chickens. In view of the necessity of utilization of serum-neutralization tests for differential diagnoses of prior exposure of chickens to Newcastle disease and infectious bronchitis, it was considered advisable to study the normal antibody level of chickens known to have been free of any previous experience with these viruses.

Minard and Jungherr[16] tested serum samples from 54 individual chickens for their capacity to neutralize a California strain of NDV in embryonating chicken eggs. Of the 54 samples, 29 were from chickens in several different states and 25 were from one flock not represented in the group of 29 samples. In the group of 29 samples, 96 per cent had 10^2 or fewer neutralizing doses. In the group of 25 samples, 68 per cent had from 10^2 to 10^6 neutralizing doses. No evidence was available regarding the spontaneous occurrence of Newcastle disease in this flock. It was concluded that the high level of neutralizing antibodies in this presumably noninfected flock suggested a latent infection. The results of the tests of the group of 29 samples in which 10^2 or fewer neutralizing doses were detected have been cited in subsequent studies as values considered to be representative of the neutralizing antibody index of normal chicken serum.

Brandly *et al.*[5] tested 46 serum samples from chickens from two different states and from normal, laboratory-stock chickens. None of these chickens or flocks had any known history of experience with Newcastle disease infection or vaccination. Of the 46 samples, 14 were negative for neutralizing antibodies, 11 had 10^2 neutralizing doses, 17 had 10^3, and four had 10^4 neutralizing doses against strain E (Hertfordshire) NDV. The extremes of 10^2 to 10^4 neutralizing doses in these presumably noninfected chickens were considered to be the "normal range." From these results, 10^3 to 10^4 neutralizing doses were considered to be the minimum for diagnosis and indication of prior Newcastle disease infection or vaccination.

Osteen and Anderson[17] reported that, with one exception, they had not found neutralization titers as high as 10^2 in composite serum samples from flocks with a negative history of Newcastle disease. These authors also considered 10^3 to 10^4 neutralizing doses to be the minimum for serologic diagnosis of prior Newcastle disease infection or vaccination.

The Committee on Transmissible Diseases of Poultry of the United States Livestock Sanitary Association[21] has also regarded 10^3 to 10^4 neutralizing doses to be the minimum for serologic diagnosis of Newcastle disease.

A report from the Bureau of Animal Industry[20] stated that ". . . birds that have never experienced the disease, or have never been vaccinated, may neutralize as much as 100 m.l.d. of virus."

Jungherr and Terrell[14] reported that chicks hatched from eggs produced by infectious bronchitis-immune breeder stock had a high level of neutralizing antibodies during the first two weeks after hatching, followed by a rapid decline and disappearance by the fifth week. Since these antibodies were transient in nature, they were considered to be the result of a naturally acquired passive immunity. Serums from random samplings of 25 chicks between the fifth and seventeenth weeks were negative for neutralizing antibodies.

Hofstad and Kenzy[12] confirmed the report of Jungherr and Terrell[14] that a high level of neutralizing antibodies could be detected in chicks hatched from eggs laid by infectious

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bronchitis-recovered hens. Infectious bronchitis could be induced, however, in 4-, 6-, 7-, and 10-day-old chicks from recovered hens. The authors consider that it is possible that complete immunity to infectious bronchitis may depend on an interaction between tissue immunity and circulating antibodies.

Page[18] used 8, 198-day-old Single Comb White Leghorn cockerels supplied by the U. S. Regional Poultry Research Laboratory for studies of the antibody response of chickens during a twelve-week period following exposure to a chicken-propagated strain of IBV. The results were evaluated by *in vitro* serum-neutralization tests as the lethal dose$_{50}$ neutralization index (l.d.$_{50}$ NI), using 5 embryonating chicken eggs per dilution. Six chickens were exposed to the virus and 2 chickens were maintained at the Regional Poultry Research Laboratory as controls. Pre-exposure serums from the 6 experimental chickens showed a range of l.d.$_{50}$ NI $10^{0.03}$ to $10^{1.38}$. Serums from the 2 control chickens showed fluctuations within the range of l.d.$_{50}$ NI $10^{0.05}$ to $10^{1.37}$ for 1 chicken and $10^{0.44}$ to $10^{1.50}$ for the other chicken during the twelve-week period.

PROCEDURE

Antigens used in the serum-neutralization tests were Michigan strain 46-967 of NDV and strain V114D of IBV. The NDV had been originally isolated from lung and tracheal material from naturally infected chickens and established and cultivated in the allantoic sac of embryonating chicken eggs. Strain V114D of IBV was originally isolated and adapted to cultivation in embryonating chicken eggs by Beaudette and Hudson.[1,6] This strain of the virus was also used in the serum-neutralization tests reported by Jungherr and Terrell,[14] Page,[18] and probably by Hofstad and Kenzy.[12]

Both viruses were capable of killing all embryos by the end of the second day after inoculation. The respective virus suspensions employed in the tests consisted of pooled allantoic fluid from embryos dead on the second day following inoculation of 10-day embryos via the allantoic sac. Aliquots of each virus suspension were stored at −45 C. until used. The suspensions were processed for use as antigens in the tests by thawing at room temperature followed by centrifugation at 2,500 revolutions per minute for twenty minutes in an International clinical centrifuge to sediment the insoluble precipitate formed on thawing. The supernatant fluid was transferred with a pipette to a glass tube which was placed in crushed ice until the fluid was used.[4]

All serums were collected by cardiac puncture from 300- to 600-day-old Single Comb White Leghorns. The serums were supplied through the courtesy of the U. S. Regional Poultry Research Laboratory, East Lansing, Mich. The chickens had been in isolation and were selected at random from pens of uninoculated controls of certain avian lymphomatosis experiments. Of the 100 chickens from which serums were collected, necropsy examinations showed that 8 had avian lymphomatosis (7 visceral and 1 neural), 5 had reproductive disorders and peritonitis, and 87 were normal. Although not all these chickens were free of pathologic alterations, they were considered as normal for the purposes of this experiment in that they had not been previously exposed to NDV or IBV.

Serial tenfold dilutions of the antigens were prepared in Difco nutrient broth.[3] The 10^{-7} to 10^{-10} dilutions of the NDV antigen were used for titration of virus activity, as previous tests had shown that the 50 per cent end point of embryo mortality could be expected between the 10^{-8} and the 10^{-9} dilutions. The 10^{-4} to

TABLE I—Comparative Frequencies of l.d.$_{50}$NI and log 10 NI of Normal Chicken Serums with IBV and NDV Antigens Using 5 Embryonating Chicken Eggs per Dilution

	IBV													
	l.d.$_{50}$NI*													
0	$10^{0.1}$	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.1	1.2	1.3	1.4
29	6	7	11	6	4	9	10	5	2	4	1	1	2	3
						log 10 NI 0								
29	4	7	11	5	1	4	3							
						log 10 NI 10^{1}								
	2			1	3	5	7	5	2	4	1		2	
						log 10 NI 10^{2}								
												1		3

	NDV													
	l.d.$_{50}$NI**													
0	$10^{0.1}$	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.1	1.2	1.3	1.4	1.5
52	2	15	5	8	4	2	3	5	3						1
						log 10 NI 0									
50	2	14	4	6	1	1			1						
						log 10 NI 10^{1}									
2		1	1	2	3	2	2	5	2						
						log 10 NI 10^{2}									1

*IBV : $\bar{X} = 10^{0.380} \pm 10^{0.0376}$, $\sigma = 10^{0.370}$.

**NDV : $\bar{X} = 10^{0.215} \pm 10^{0.0282}$, $\sigma = 10^{0.272}$.

10^{-7} dilutions of the IBV antigen were used for titration of virus activity, as the 50 per cent end point of embryo mortality was expected to be between the 10^{-5} and the 10^{-6} dilutions.

Equal parts of undiluted serum and the diluted antigen were mixed and incubated at room temperature for approximately forty-five minutes before injection into embryonating chicken eggs. The serums were tested over the range of the 10^{-5} to the 10^{-9} dilutions of NDV antigen and over the range of the 10^{-2} to the 10^{-6} dilutions of the IBV antigen.

To compensate for the increased dilution of the antigens when mixed with equal parts of serum, quantitative titrations of virus activity were made by mixing equal parts of each antigen dilution with the diluent. In all instances, the same volumes were used with the serum-antigen and diluted antigen-diluent mixtures.

Five 10-day embryonating chicken eggs were used per dilution and 0.1 cc. of inoculum was injected via the allantoic sac.[5] All incubation was at 99-99.5 F., wet bulb 86-88 F., in an electric, forced-draft incubator. Embryo mortality within the first twenty-four hours following inoculation was considered to be due to non-specific causes and was not included in the computations of embryo mortality rates. The eggs were incubated for six days following inoculation.

The titers of the antigens and of the serums, expressed as $l.d._{50}$, were calculated according to the 50 per cent end point method of Reed and Muench,[9] utilizing cumulative frequencies of embryo mortality and survival. Calculations of the titers were made to the centile and rounded off to the decile. The difference between the antigen titer and the serum titer was indicated as the $l.d._{50}$ NI, of which the antilog of the reciprocal was the numbers of neutralizing doses in 0.05 cc. of the undiluted serum.

For comparative purposes, the NI was also computed on the basis of the tenfold dilutions only, in which the titers of the antigen and of the serums were considered to be the highest dilution of the antigen in which 50 per cent or more of the 5 eggs inoculated per dilution were killed by the virus. The difference between the antigen titer and the serum titer was indicated as the log 10 neutralization index (log 10 NI) of which the antilog of the reciprocal was the numbers of neutralizing doses in 0.05 cc. of the undiluted serum.

RESULTS AND DISCUSSION

The results of the 100 serum-neutralization tests are shown in table 1.

With the IBV antigen, the $l.d._{50}$ NI was 0 for 29 serums, $10^{0.1}$ to $10^{1.0}$ for 64 serums, and $10^{1.1}$ to $10^{1.4}$ for seven serums. The $\overline{X}$ was $10^{0.389} \pm 10^{0.0376}$ as calculated from $\overline{X} = \overline{X}^1 + Ci \frac{\Sigma(fd)}{N}$, with 0-$10^{0.1}$ as the lowest class limits and class intervals of $10^{0.1}$. The σ was $10^{0.376}$ as calculated from $\sigma = Ci \sqrt{\frac{\Sigma(fd^2)}{N} - \left(\frac{\Sigma(fd)}{N}\right)^2}$. These data would indicate that with serum-neutralization tests of normal serums, the $l.d._{50}$ NI of 68 per cent ($\overline{X} + \sigma$) of the serums would not be expected to exceed $10^{0.765} \pm 10^{0.0376}$, 95.5 per cent ($\overline{X} + 2\sigma$) $10^{1.041} \pm 10^{0.0376}$, and 99.7 per cent ($\overline{X} + 3\sigma$) $10^{1.517} \pm 10^{0.0376}$. The maximum numbers of neutralizing doses, antilog of the $l.d._{50}$ NI, would be approximately 6, 15, and 36, respectively.

Assessment of the serums with the IBV antigen by the log 10 NI showed that the log 10 NI was 0 for 64 serums, 10^1 for 32 serums, and 10^2 for four serums. Of the 64 serums that were log 10 NI 0, 29 were $l.d._{50}$ NI 0 and 35 were within the range of $l.d._{50}$ NI $10^{0.1}$ to $10^{0.7}$. The 32 serums that were log 10 NI 10^1 were within the range of $l.d._{50}$ NI $10^{0.1}$ to $10^{1.3}$, with 28 of the serums within the range of $l.d._{50}$ NI $10^{0.4}$ to $10^{1.1}$ There was an overlap of the log 10 NI 0 and 10^1 serums in the range of $l.d._{50}$ NI $10^{0.4}$ to $10^{0.7}$ The four serums of log 10 NI 10^2 occurred in the $l.d._{50}$ NI $10^{1.2}$ and $10^{1.4}$.

These results indicate that if the serums had been evaluated only on the basis of the log 10 NI, 35 per cent of the serums would have been classified as negative but, on the basis of the $l.d._{50}$ NI, these serums would have contained from one to five neutralizing doses. Similarly, 32 per cent of the serums on the log 10 NI would have been classified as having ten neutralizing doses but, on the $l.d._{50}$ NI, these serums would have contained from one to 20 neutralizing doses (28% would have contained from 3 to 13 neutralizing doses). There would have been an overlap of the range from three to five doses in the log 10 NI 0 and the log

10 NI 10^1 neutralizing doses. The log 10^2 NI indicated that 4 per cent of the serums contained 100 neutralizing doses, whereas, on the $l.d._{50}$ NI, these samples contained from 16 to 25 neutralizing doses.

Summation of these data show that on the log 10 NI, 64 per cent of the serums would have been classified as negative, 32 per cent would have contained ten neutralizing doses, and 4 per cent would have contained 100 neutralizing doses. Assessment of these serums on the $l.d._{50}$ NI would indicate that 29 per cent were negative, 64 per cent contained from one to ten neutralizing doses, and 7 per cent contained from 13 to 25 neutralizing doses.

Page's[18] data on serums from 6 chickens prior to exposure to IBV showed that the $l.d._{50}$ NI ranged from $10^{0.63}$ to $10^{1.28}$. Evaluation of these serums on the log 10 NI showed that the log 10 NI was 10^1 in the $l.d._{50}$ NI range of $10^{0.63}$ to $10^{0.85}$, and 10^2 in the range of $10^{0.94}$ to $10^{1.28}$. Serum from 1 control chicken fluctuated within the range of $l.d._{50}$ NI $10^{0.05}$ to $10^{1.37}$ during a twelve-week period. The log 10 NI was negative in the $l.d._{50}$ NI range of $10^{0.05}$ to $10^{0.47}$, 10^1 in the $10^{0.66}$ to $10^{0.84}$, and 10^2 for $10^{1.37}$. Serum from the other control chicken ranged from $l.d._{50}$ NI $10^{0.44}$ to $10^{1.53}$. The log 10 NI was negative for $l.d._{50}$ NI $10^{0.44}$ to $10^{0.63}$, 10^1 for $10^{0.67}$ to $10^{0.85}$, and 10^2 for $10^{1.37}$ to $10^{1.53}$. These data are similar to those reported for the present study.

With the NDV antigen, the $l.d._{50}$ NI was 0 for 52 serums, $10^{0.1}$ to $10^{1.0}$ for 47 serums, and $10^{1.5}$ for one serum. The $\overline{X}$ was $10^{0.215} \pm 10^{0.0262}$ and the σ was $10^{0.262}$. These data would indicate that with serum-neutralization tests of normal serums, the $l.d._{50}$ NI of 68 per cent $(\overline{X} + \sigma)$ of the serums would not be expected to exceed $10^{0.477} \pm 10^{0.0262}$, 95 per cent $(\overline{X} + 2\sigma)$ $10^{0.739} \pm 10^{0.0262}$, and 99.7 per cent $(\overline{X} + 3\sigma)$ $10^{1.001} \pm 10^{0.0262}$. The maximum numbers of neutralizing doses, antilog of the $l.d._{50}$ NI, would be approximately 3, 6, and 11, respectively.

Assessment of the serums with the NDV antigen by the log 10 NI showed that the log 10 NI was 0 for 79 serums, 10^1 for 20 serums, and 10^2 for one serum. Of the 79 serums that were log 10 NI 0, 52 were also $l.d._{50}$ NI 0. Of these 52 serums, 50 were log 10 NI 0 and 2 were log 10 NI 10^1. The 79 serums that were log 10 NI 0 were within the range of $l.d._{50}$ NI 0 to $10^{0.9}$. The 20 serums that were log 10 NI 10^1 were within the range of $l.d._{50}$ NI 0 to $10^{0.9}$, with 18 of the serums within the $l.d._{50}$ NI range of $10^{0.2}$ to $10^{0.9}$. The one serum of log 10 NI 10^2 was $l.d._{50}$ NI $10^{1.5}$.

These results indicate that if the serums had been evaluated only on the basis of the log 10 NI, 79 per cent would have been classified as negative but, on the basis of the $l.d._{50}$ NI, these serums would have contained from zero to eight neutralizing doses. Similarly, 20 per cent of the serums on the log 10 NI would have been classified as having ten neutralizing doses but, on the $l.d._{50}$ NI, these serums would have contained from zero to eight neutralizing doses (18% would have had from 2 to 8 neutralizing doses). The log 10 NI indicated that one serum contained 100 neutralizing doses but, on the basis of the $l.d._{50}$ NI, this serum contained 32 neutralizing doses.

Summation of these data show that on the log 10 NI, 79 per cent of the serums would have been classified as negative, 20 per cent would have contained ten neutralizing doses, and 1 per cent would have contained 100 neutralizing doses. Assessment of these serums on the $l.d._{50}$ NI would indicate that 52 per cent were negative, 47 per cent contained from one to ten neutralizing doses, and 1 per cent contained 32 neutralizing doses.

The data for the serum-neutralization tests indicate that when 5 eggs are used per dilution, the $l.d._{50}$ NI is a more critical means of evaluating the neutralizing capacity of the serum than the log 10 NI. The $l.d._{50}$ NI is the result of a statistical analysis of the cumulative frequencies of the embryo mortality and survival data and takes into consideration variables which might be encountered with the embryos used for the test. The log 10 NI permits detection of only tenfold differences in titers of the antigen and of the serum and does not take into consideration variables of mortality above 50 per cent per dilution. With the log 10 NI, the mortality per dilution may range from 60 per cent to 100 per cent without exerting any influence on the evaluation of the titer. Such variables exert a marked influence when the titer is calculated according to the $l.d._{50}$ NI.

SUMMARY

In vitro serum-neutralization tests with infectious bronchitis virus (IBV) and Newcastle disease virus (NDV) antigens were conducted with serums from 100, 300- to 600-day Single Comb White Leghorn cockerels known to have been free of any previous exposure to either of these viruses. Five embryonating chicken eggs were used per dilution for the tests and the results were evaluated as the lethal dose$_{50}$ neutralization index (l.d.$_{50}$ NI) and the log 10 neutralization index (log 10 NI).

With the IBV antigen, the l.d.$_{50}$ NI was 0 for 29 serums, $10^{0.1}$ to $10^{1.0}$ for 64 serums, and $10^{1.1}$ to $10^{1.4}$ for seven serums. The $\overline{X}$ was $10^{0.389} \pm 10^{0.0376}$ and the σ was $10^{0.376}$. The log 10 NI was 0 for 64 serums, 10^{1} for 32 serums, and 10^{2} for four serums. The serums of the log 10 NI 0 occurred in the range of l.d.$_{50}$ NI 0 to $10^{0.7}$, the 10^{1} to the l.d.$_{50}$ NI $10^{0.1}$ to $10^{1.3}$, and the 10^{2} to the l.d.$_{50}$ NI of $10^{1.2}$ and $10^{1.4}$.

With the NDV antigen, the l.d.$_{50}$ NI was 0 for 52 serums, $10^{0.1}$ to $10^{1.0}$ for 47 serums, and $10^{1.5}$ for one serum. The $\overline{X}$ was $10^{0.215} \pm 10^{0.0262}$ and the σ was $10^{0.262}$. The log 10 NI was 0 for 79 serums, 10^{1} for 20 serums, and 10^{2} for one serum. The serums of log 10 NI 0 and 10^{1} occurred in the range of l.d.$_{50}$ NI 0 to $10^{0.9}$. The log 10 NI 10^{2} serum was l.d.$_{50}$ NI $10^{1.5}$.

From the data presented, the l.d.$_{50}$ NI of 99.7 per cent ($\overline{X} + 3\sigma$) of normal chicken serums would not be expected to exceed $10^{1.517} \pm 10^{0.0376}$ with IBV antigen, and $10^{1.001} \pm 10^{0.0262}$ with NDV antigen, or a maximum of 36 and 11 neutralizing doses, respectively.

The l.d.$_{50}$ NI is considered to be more accurate than the log 10 NI for evaluation of *in vitro* serum-neutralization tests with IBV and NDV antigens when 5 eggs are used per dilution.

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A Study of Sulfaquinoxaline and Sulfamethazine in Fowl Typhoid Among Breeder Turkeys

W. A. BONEY, JR., D.V.M., M.S.

College Station, Texas

FOWL TYPHOID is primarily a disease of chickens and turkeys. The acute form produces a septicemia and usually results in high mortality. The disease is caused by *Salmonella gallinarum* and the acute form in turkey poults is clinically indistinguishable from other Salmonella species.

When the disease is present in the chronic form, the organism usually is not present in the circulating blood. However, bacteriologic examinations made on reactors to the pullorum test often reveal *S. gallinarum* in the heart, ovary, oviduct, and caseous material which may be present in the abdominal cavity. The chronic form of the infection can be spread and transmitted through the egg[1] and infected feces as is pullorum disease. The organism is frequently isolated from young poults as well as from growing and mature turkeys. The etiology, symptoms, and pathology of this disease are adequately described by Hinshaw[2] and Bushnell.[3]

This paper deals with a flock of breeder turkeys, that were classified as U. S. Pullorum Clean two weeks prior to the time they became infected with fowl typhoid. Infection probably came from contaminated surface tank water that was being used as an emergency supply.

REVIEW OF LITERATURE

Pomeroy *et al.*,[4] in reporting on sulfonamide therapy, stated that sulfathiazole, sulfadiazine, and sulfamerazine were not effective in treating fowl typhoid in experimentally infected chickens. They also reported that sulfaquinoxaline was toxic for chicks when incorporated in the mash at the rate of 0.5 and 1.0 per cent, but noted that there was some indication of the control of pullorum disease. Bankowski[5] reported that sulfamethazine, 0.5 per cent in the mash for six days or in the drinking water at the rate of 0.2 per cent for three days, showed adverse effects on egg production of chickens. He stated that egg production reached the lowest level seven to nine days after the beginning of medication and that it required three and one-half weeks for the birds to resume normal egg production after medication was stopped. Delaplane[6] found that sulfaquinoxaline added to the mash at the rate of 0.1 or 0.05 per cent controlled chronic fowl cholera, but stated that laying hens could not tolerate these levels of the drug in the mash continuously. He found that egg production began to decline eight days after medication was begun, but he pointed out that mature laying hens could tolerate the drug, continuously, in the mash at a 1 : 3,000 (0.033%) level.

Peterson[7] reported that sulfaquinoxaline, when given in the drinking water at the rate of 0.05 per cent and 0.025 per cent, controlled acute fowl cholera in turkeys, and that sulfamethazine at the rate of 0.2 per cent in the mash likewise gave good control of this disease in experimental chickens. Kiser *et al.*[8] stated that sulfamethazine at the rate of 0.5 and 1.0 per cent in the mash and 0.1 per cent in the drinking water gave excellent control of acute fowl cholera.

FIELD TRIALS WITH SULFAMETHAZINE

On Dec. 22, 1948, fowl typhoid was diagnosed on farm A in a flock of turkeys consisting of approximately 7,000 hens and toms, classified as U.S. Pullorum Clean, which had been selected for breeding purposes. Approximately, 1,500 of these birds, including 976 pedigree toms, died between Dec. 22, 1948, and Jan. 20, 1949. When the diagnosis was established, the birds were given sulfamethazine in the drinking water at the rate of 1 : 1,000 (0.1%) for five days, followed by an intermittent schedule of two days on the drug and three days off, without apparent beneficial results. On January 10, each turkey was injected intraperitoneally with 4 cc. of a 25 per cent solution of sulfamethazine. The mortality was definitely reduced for three to four days, and another injection, given on January 15, prevented further mortality for another four to five days.

Between Dec. 28, 1948 and Jan. 20, 1949, these turkeys were blood tested three times, using the standard tube-agglutination pullorum test. All reactors were removed to what

From the Texas Agricultural Experiment Station, and School of Veterinary Medicine, Texas A. & M. College, College Station.

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was designated as the reactor pen. This procedure was valueless in controlling the disease. Often, birds with a negative test would die from the disease by the time the test was completed. Following each test, the number of reactors increased and the mortality continued. Rectal temperatures were taken twice on all birds. Those birds having a temperature above 108 F. were segregated, and sulfamethazine was administered intraperitoneally. The mortality in the group segregated because of fever was held to a minimum, but this procedure was of little value on a flock basis, since many birds with temperatures below 108 F. died within twenty-four hours.

The owner was advised to dispose of the flock, but he desired to save some of the valuable breeding stock. Consequently, sulfaquinoxaline therapy was initiated as soon as sufficient quantities could be obtained.

FIELD TRIALS WITH SULFAQUINOXALINE

On Jan. 10, 1949, fowl typhoid was diagnosed in a flock of 456 selected breeder turkeys on farm B, 5 miles from farm A. The water supply was a surface tank.

Since the results on farm A with sulfamethazine had not been encouraging, sulfaquinoxaline was given in the drinking water to the flock on farm B.

The source of the water was not changed. By January 10, 30 turkeys had died. On January 12 and 13, the drug was given in the drinking water at the rate of 1 : 2,500 (0.04%), and thereafter it was administered continuously at the rate of 1 : 5,000 (0.02%). From January 11 to 13, 18 more turkeys died. Only five deaths were reported the following week. No more deaths were reported until approximately one month later, when birds had been without the medicament for five days. Sulfaquinoxaline was again added to the water at the rate of 1 : 2,500 (0.04%) for two days, and thereafter the flock was given a concentration of 1 : 5,000 (0.02%) continuously for the remainder of the laying season (approxi-

TABLE 1—Effects of Sulfaquinoxaline on Mortality, Egg Production, Fertility, Hatchability, and Spread of Fowl Typhoid Among Breeder Turkeys

	No. hens		Weight (lb.)		Per cent					
Lot	Start	Finish	Start	Finish	Production	Fertile	Fertile hatch	Total hatch	Deaths	New reactors
Control—no treatment										
1	14	14	251.8	234.2	51	79	85	67	6.25	7.14
2	14	12	224.5	201.5	62	58	82	48	12.50	7.14
3	14	12	206.1	194.7	57	62	76	47	18.75	21.40
Total	42	38	682.4	630.4	57	66	81	54	12.50	11.90
Weight loss				7.6%						
Sulfaquinoxaline 1 : 8,000 in water										
4	14	14	251.3	232.2	55	65	81	52	0.00	0.00
5	14	14	242.6	232.5	50	27	81	22	0.00	14.28
6	14	14	245.7	228.0	48	85	80	68	0.00	0.00
Total	42	42	739.6	692.7	51	59	81	47	0.00	4.76
Weight loss				6.3%						
Sulfaquinoxaline 1 : 6,000 in water										
7	14	14	255.0	240.8	59	19	79	15	0.00	0.00
8	14	13	228.0	216.3	55	76	84	64	0.00	0.00
9	14	11	198.0	179.6	50	57	82	47	12.50	0.00
Total	42	38	681.0	636.7	55	50	82	41	4.20	0.00
Weight loss				6.5%						
Sulfaquinoxaline 1 : 4,000 in water										
10	14	14	258.6	245.3	58	84	77	65	0.00	0.00
11	14	14	257.8	226.6	42	69	75	52	0.00	28.57
12	14	14	257.7	229.8	52	77	85	65	0.00	0.00
Total	42	42	774.1	701.7	51	78	79	61	0.00	9.52
Weight loss				9.3%						
Sulfaquinoxaline 1 : 2,000 in water										
13	14	14	237.9	228.9	45	74	74	55	0.00	28.57
14	14	14	248.1	231.2	48	31	52	16	0.00	21.40
15	14	13	231.9	211.6	47	79	78	61	0.00	7.14
Total	42	41	717.9	671.7	47	61	72	44	0.00	19.04
Weight loss				6.4%						
Total 15 lots			3,595.0	3,333.2	52	63	79	49	3.34	9.04
Weight loss				7.3%						

Two nonreactor toms added to each pen, giving total of 16 birds per pen.

mately 100 days). No further mortality due to fowl typhoid was observed.

On January 21, the large flock on farm A was given sulfaquinoxaline in the drinking water in a concentration of 1 : 2,500 (0.04%) for three days, after which it was reduced to 1 : 5,000 (0.02%). Within four days, the mortality was decidedly reduced. The flock was kept on the 1 : 5,000 (0.02%) solution until February 22, when a slight increase occurred in the mortality, and the concentration was again increased to 1 : 4,000 (0.025%). This level of sulfaquinoxaline did not completely prevent mortality and, on February 27, the amount was raised to 1 : 2,500 (0.04%) for two days, then reduced to 1 : 3,000 (0.033%) until March 17. From March 18 to the end of the laying season on June 4, sulfaquinoxaline was given at the rate of 1 : 4,000 (0.025%) in the drinking water. The mortality throughout the season from all causes was less than 1 per cent each week. Early results were so encouraging that the owner confined 336 of the best turkeys in groups of 14 in 24 breeder tents, and administered sulfaquinoxaline in the water at the rate of 1 : 4,000 (0.025%) for the entire laying season. Mortality was much less than usual.

EXPERIMENTAL TRIALS

Since favorable results were obtained at the beginning of the field trials with sulfaquinoxaline, and since information regarding the use of the drug in normal turkeys and fowl typhoid-infected turkeys was not available, controlled experiments were initiated to determine the effect of administration of different levels of the drug to breeder turkeys on a continuous basis. After a tentative plan was formulated for the sulfaquinoxaline experiment, it was also decided to arrange similar trials for the use of sulfamethazine.

The objectives were to determine: (1) the concentrations of sulfaquinoxaline and sulfamethazine that laying turkey hens and turkey toms could tolerate in the drinking water; (2) the effects of sulfaquinoxaline and sulfamethazine on fowl typhoid infection in adult breeder turkeys exposed under natural conditions; and (3) the effect of sulfaquinoxaline and sulfamethazine on egg production, eggshell texture, fertility, and hatchability of eggs produced by turkey hens given different levels of the two drugs continuously. (At the same time, it was decided to investigate the possible egg transmission of the disease.)

Female turkeys were selected from the large flock on farm A and divided into 24 lots. Each lot contained 2 reactor hens and 12 nonreactor hens, and to each there were added 2 nonreactor toms from a flock free of the disease. The nonreactor hens had been negative on three consecutive pullorum tests not less than seven or more than fourteen days apart. Each lot of 16 turkeys was confined in a tent 14 ft. sq. on sandy soil, and the tents were placed 20 ft. apart. The hens and toms were identified by leg bands. Individual weights were recorded at the beginning and end of the experiment.

At the beginning of the experimental period on February 22, the hens were selected and mated for production of hatching eggs. Electric lights were used to hasten sexual maturity and initiation of egg production. The birds were tested every two weeks in order to determine the rate of spread of the disease. Mortality records were kept and the cause of death was determined by laboratory and bacteriologic examination. The eggs were candled at from eighteen to twenty-five days of incubation, and all infertile eggs, dead germs, and dead embryos were examined bacteriologically. All eggs that failed to hatch were treated in a similar manner.

With the exception of the males, all birds had received sulfamethazine orally and parenterally as well as sulfaquinoxaline in the water for one month prior to their selection for the experiment.

Beginning February 22, sulfaquinoxaline was added to the drinking water of separate experimental lots of turkeys at the rate of 1 : 2,000 (0.05%), 1 : 4,000 (0.025%), 1 : 6,000 (0.0166%), and 1 : 8,000 (0.0125%) for forty-nine days. Sulfamethazine was given in the drinking water to other experimental lots at the level of 1 : 1,000 (0.1%), 1 : 3,000 (0.033%), and 1 : 5,000 (0.02%) for an equal period. Three lots of turkeys were used for each drug level. The experiments embraced a total of 384 turkeys.

Another flock of 1,000 mated hens which was not affected with fowl typhoid and received no medication served as additional controls. These turkeys, of the same breeding as the experimental birds, were on a separate farm under the same management but with different attendants, and so provided comparative data on egg production, fertility, and hatchability.

The infected flock from which the experimental birds were selected was kept in semiconfinement in tents. This group of birds was designated as the commercial egg flock.

RESULTS AND DISCUSSION

Groups of turkeys from an infected field flock (farm A) were selected for treatment with different concentrations of sulfaquinoxaline or sulfamethazine in the drinking water continuously for forty-nine days. At the beginning of the experiment, considerable difficulty was anticipated among birds in those lots receiving the higher concentrations of the two drugs,

since information available indicated that toxicity would develop and would become evident by the appearance of various disorders associated with egg production.

Tables 1 and 2 show that the turkeys tolerated sulfaquinoxaline in the water in various concentrations from 1 : 8,000 to 1 : 2,000 for forty-nine days without apparent adverse effects on egg production, fertility, and hatchability. Similarly, toxic effects were not seen in birds on the different concentrations of sulfamethazine, 1 : 5,000, 1 : 3,000, and 1 : 1,000, in drinking water.

Bacteriologic examinations of the birds that died in this experiment revealed that the cause of death in each instance was fowl typhoid. In several individuals, reproductive disorders were present but, since *S. gallinarum* was isolated from these cases, fowl typhoid was considered the primary cause of death.

In the three control pens, six deaths resulted from fowl typhoid. Five of the birds were nonreactors at the beginning of the experiment. Only 1 of the original reactors placed in the control pens died of the disease.

There were no deaths among three lots of birds on sulfaquinoxaline-treated drinking water at the rate of 1 : 8,000. Among the three lots on sulfaquinoxaline at the rate of 1 : 6,000, there were two deaths, but mortality was absent among the lots receiving the 1 : 4,000 and 1 : 2,000 concentrations. Neither toxic manifestations nor faulty eggshell texture was observed in any of the lots.

If only one pen of birds (No. 5, 7, or 14) had been used for this experiment, the results would have indicated that sulfaquinoxaline had a disastrous effect on fertility and hatchability. However, in each instance, two other pens received the same concentrations, and the results among them indicate that the drug was not responsible. When the toms in these pens were replaced by potent toms, later in the season, the fertility and hatchability of the eggs was comparable to those of other groups.

Lots 10, 11, and 12, which received sulfaquinoxaline in the water at the rate of 1 : 4,000, had the highest total hatch (61%). Lots 13, 14, and 15, which received sulfaquinoxaline in the water at the rate of 1 : 2,000, had an average hatch of 44 per cent. If the results obtained from lot 14 were discarded, the average would be 58 per cent hatch for the other two lots. Comparatively, this would be considered a good hatch, and it indicates that levels of sulfaquinoxaline as high as 1 : 2,000 may

TABLE 2—Effects of Sulfamethazine on Mortality, Egg Production, Fertility, Hatchability, and Spread of Fowl Typhoid Among Breeder Turkeys

	No. hens		Weight (lb.)		Per cent					
Lot	Start	Finish	Start	Finish	Production	Fertile	Fertile hatch	Total hatch	Deaths	New reactors
Sulfamethazine 1 : 5,000 in water										
16	14	13	224.3	211.0	59	78	78	61	6.25	0.00
17	14	14	251.0	231.3	56	72	76	55	0.00	0.00
18	14	12	211.2	193.5	46	65	90	59	12.50	7.14
Total	42	39	686.5	635.8	53	72	80	58	6.25	2.38
Weight loss				7.4%						
Sulfamethazine 1 : 3,000 in water										
19	14	12	220.0	204.3	52	73	77	57	6.25	7.14
20	14	13	224.8	214.0	45	70	79	55	6.25	7.14
21	14	13	230.7	212.9	51	70	85	59	6.25	14.28
Total	42	38	675.5	631.2	49	71	80	57	6.25	9.52
Weight loss				6.5%						
Sulfamethazine 1 : 1,000 in water										
22	14	13	226.7	213.1	49	66	80	52	6.25	14.28
23	14	13	241.2	211.8	52	82	78	64	0.00	21.42
24	14	12	222.5	195.5	44	66	81	53	12.50	7.14
Total	42	38	690.4	620.4	48	72	79	57	6.25	11.90
Weight loss				10.1%						
Total 9 lots			2,052.4	1,887.4	50	72	80	57	6.25	7.93
Weight loss				8.0%						

Two nonreactor toms added to each pen, giving total of 16 birds per pen.

be used for longer periods than heretofore have been considered safe.

Our observations on the field trials of sulfamethazine with respect to controlling mortality were verified by the experimental data. There was a 6.25 per cent mortality with three levels of sulfamethazine as compared to 1.0 per cent mortality with four levels of sulfaquinoxaline, all of which occurred in the three lots receiving the latter drug at a level of 1 : 6,000.

During the hatching season, the eggs which were examined bacteriologically were produced by both the experimental and commercial egg flocks. They were collected from the incubators at the time of candling and at the end of each hatch. They consisted of infertile eggs, dead germs, dead embryos with the shell intact, and dead embryos with the shell pipped. Effort was made to separate the eggs with respect to the individual drugs, but not to the various concentrations. The number of eggs examined bacteriologically from the experimental and commercial egg flocks were 3,572 and 400, respectively. *Salmonella gallinarum* was isolated from eggs produced by the experimental birds without indication that it was more prevalent in eggs from hens on either of the two drugs. The organisms were isolated from two pools of 20 infertile and dead germ eggs produced by hens receiving sulfaquinoxaline; from one pool of ten infertile and dead germ eggs from hens receiving sulfamethazine; also from two pools of ten eggs with dead embryos from hens receiving sulfaquinoxaline; from three pools of ten eggs from hens receiving sulfamethazine; from one pool of ten dead embryos with pipped shells from hens receiving sulfaquinoxaline, and from one pool of 15 dead embryos with pipped shells from hens receiving sulfamethazine.

Table 3 summarizes the results that were obtained from the commercial flocks, one of which was infected with fowl typhoid and received sulfaquinoxaline at the rate of 1 : 4,000 in the drinking water continuously and one which was noninfected and nonmedicated.

The owner of farm A, operating a large hatchery (hatchery A), was able to set eggs from the treated experimental lots as well as eggs from the sulfaquinoxaline-medicated commercial flock. Another hatchery (hatchery Y) accepted shipments of eggs from the infected sulfaquinoxaline-medicated commercial flock, and a third hatchery (hatchery X) accepted eggs from the noninfected and nontreated commercial flock. Table 3 shows that hatchery A set 26,735 eggs from the treated flock (not including experimental flock eggs) which had a fertility of 73 per cent and that hatchery Y set 46,000 similar eggs, later in the season, and had a fertility of 62 per cent. These figures compared favorably with the results of hatchery X, where a fertility of 64 per cent on 38,600 eggs was recorded. These reports indicate that sulfaquinoxaline, when given in water at the rate of 1 : 4,000, did not adversely affect fertility.

By comparing results of the experimental birds in tables 1 and 2, it will be seen that the average total fertility for the eggs from the three experimental groups was 66 per cent. This compares favorably with nonmedicated commercial flocks. The experimental birds on the four levels of sulfaquinoxaline had a total average fertility of 61.5 per cent and those on three levels of sulfamethazine had a total average fertility of 71.0 per cent.

Attention is called to the performance of the three pens given sulfamethazine in the drinking water at the rate of 1 : 1,000 (0.1%), which had a 72 per cent fertility, a 79 per cent hatch of fertile eggs, and a 57 per cent total hatch of all eggs set.

Mortality in the control hens was 12.5 per cent, while that of the sulfamethazine groups was 6.25 per cent, as compared to

TABLE 3—Comparison of Fertility in Turkey Eggs from Commercial Laying Flocks Treated and Untreated with Sulfaquinoxaline

	Treated* flock Farm A hatchery		Untreated flock Hatchery X	
	Total eggs	Fertile (%)	Total eggs	Fertile (%)
March 11	3,240	65	5,600	73
March 15	3,007	70	2,400	67
March 18	4,440	72	2,200	64
March 22 to 29	16,048	76	6,800	70
Total March	26,735	73	17,000	70
	Hatchery Y		Hatchery X	
March 30 to April 2	5,800	70	4,600	50
April 3 to 9	7,400	68	4,200	64
April 10 to 16	7,400	63	3,800	67
April 17 to 22	5,800	59	2,600	66
April 23 to May 1	9,000	57	4,200	62
May 2 to 11	9,600	58	2,200	55
Total March 30 to May 11	46,000	62	21,600	61
Total season	72,735	66	38,600	64

*Sulfaquinoxaline in drinking water at the rate of 1 : 4000 continuously.

1.0 per cent among those on sulfaquinoxaline.

Sulfamethazine or sulfaquinoxaline did not prevent the spread of fowl typhoid, as shown by the occurrence of 26 new reactors in the two experimental groups. Six of these new reactors had lost their titer completely by the time of the latest test. Neither of the drugs appeared to prevent the transmission of the organisms through the eggs.

However, sulfaquinoxaline apparently reduced greatly the mortality caused by fowl typhoid when the infected flock was given the drug in water at the rate of 1 : 2,500 (0.04%) for two to three days, then reduced to 1 : 4,000 (0.025%) and given continuously. These levels were not toxic and served to check mortality in an infected flock until a market was found. The infected commercial flock received the drug for a 134-day period, without showing toxic effects. It is not recommended for use in breeder flocks other than to stop the mortality until a market is found for the clinically normal birds. Buyers of birds in which fowl typhoid is being suppressed by sulfonamide administration should be warned that the birds should be slaughtered immediately to avoid further losses from the disease.

Several groups of poults which were the progeny of typhoid-infected hens were started on turkey starter mash containing 0.0125 per cent sulfaquinoxaline. Since fowl typhoid caused a heavy mortality during the first week, sulfaquinoxaline was also added to water at the rate of 1 : 8,000 (0.0125%). The mortality was overcome within the week. There were no indications that livability of the poults was affected by the medication of the parent stock.

SUMMARY

Results of field trials are reported with sulfaquinoxaline and sulfamethazine in the treatment of fowl typhoid infection among turkeys. Attention is directed toward the effects of continuous medication of the drinking water upon the disease, egg production, fertility, and hatchability.

Sulfamethazine administered as 1 : 1,000 (0.1%) of the drinking water did not control losses from fowl typhoid in field flocks. When administered intraperitoneally, it reduced the mortality temporarily but, in order to maintain the prophylactic effect, it was necessary to repeat the injections every four or five days.

Three levels of sulfamethazine, 1 : 5,000 (0.02%), 1 : 3,000 (0.033%), and 1 : 1,000 (0.01%) were not toxic and had no effect on water consumption, egg production, fertility, or hatchability of eggs, when given continuously in the drinking water for forty-nine days. The average mortality in the three groups on the three levels of sulfamethazine was 6.25 per cent as compared to 12.5 per cent in the controls.

Sulfaquinoxaline effectively reduced the mortality in two field outbreaks of fowl typhoid among turkeys when given in the drinking water at the rate of 1 : 2,500 (0.04%) for two days, and then continued at levels of 1 : 4,000 (0.025%) in one flock and 1 : 5,000 (0.02%) in the other flock.

Four levels of sulfaquinoxaline, 1 : 8,000 (0.0125%), 1 : 6,000 (0.0166%), 1 : 4,000 (0.025%), and 1 : 2,000 (0.05%), did not adversely affect water consumption, egg production, or fertility and hatchability of eggs. No toxicity was observed with any of the concentrations used, and the mortality in the experimental groups for the forty-nine-day period was approximately 1.0 per cent.

Periodic tests revealed that neither sulfaquinoxaline nor sulfamethazine prevented further spread of the disease in the infected flocks. During the period of forty-nine days, one half of the original reactor birds lost their titers completely; others lost it for one or two tests, and then regained it.

Of the original 288 nonreactor hens, 26 became reactors during the experiment, but 6 of them were negative on the last test.

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Infectious Bronchitis

H. VAN ROEKEL, D.V.M., Ph.D.; M. K. CLARKE, B.A., M.S.; K. L. BULLIS, D.V.M., M.S.; O. M. OLESIUK, B.A., M.S.; F. G. SPERLING, V.M.D.

Amherst, Massachusetts

INFECTIOUS BRONCHITIS was first recognized as a disease entity of chickens approximately nineteen years ago. In 1931, Schalk and Hawn[5] described this malady as an acute, viral, respiratory disease in baby chicks. Through the years, other investigators have reported upon various aspects of the disease. Since an extensive review of the literature will be published elsewhere by the authors,[7] only references pertinent to this paper are mentioned.

The distribution of this disease is not accurately known, but it is believed to be widespread in this country. It has also been reported in Canada and England. The disease is of great economic importance to the poultry industry, especially in areas where poultry is concentrated. The losses occur chiefly through chick mortality and retardation in growth, and a loss in egg production and egg quality in laying flocks. In some areas, the losses from infectious bronchitis exceed those from other avian respiratory infections, including Newcastle disease.

The need for effective control measures of the disease has long been recognized. Various aspects of the disease have been studied at this laboratory during the past ten years and the results are presented.

INCUBATION AND COURSE OF THE DISEASE

Early investigators stressed the fact that the disease was highly contagious and spread with great rapidity. This observation applied to experimental laboratory birds as well as to birds in commercial flocks.

In a series of 118 inoculation trials involving 377 susceptible chickens ranging in age from 2 to 35 weeks, a chicken-propagated virus was employed for the transmission studies. The birds were inoculated intratracheally with tracheal swabbings taken from living infected birds, or with tracheal scrapings suspended in physiologic saline or broth and applied with a swab, intratracheally. The birds were checked daily for symptoms. Table 1 reveals that respiratory symptoms were observed within a minimum of twenty-four hours and a maximum of six days after inoculation. The greater percentage of birds showed the first symptoms on the second, third, and fourth days after inoculation. In some trials, respiratory râles appeared uniformly early after inoculation, whereas in other trials, this symptom was delayed. Even among birds in the same trial, considerable variation in appearance of symptoms was observed. It would appear that a variation in the resistance to infectivity of the virus exists among birds. On the other hand, the degree of infectivity of the virus may have been at a level which failed to produce symptoms at a uniform and constant rate. It is clearly evident from the results that the disease has a very short incubation period, which presents a problem in natural outbreaks from the standpoint of establishing barriers against the spread of the disease within the flock on the same premises. When the disease appears in a flock, the infection, as a rule, will spread to all susceptible birds within a few days. Practical preventive measures instituted against the spread of the disease on a farm usually fail.

The course of the disease was studied in 192 artificially infected chickens ranging in age from 1 to 32 or more weeks. The majority manifested symptoms for one to eleven days. A few birds exhibited symptoms for fourteen to twenty-nine days. Similar observations were made also in natural outbreaks. In some flocks, individuals continued to show symptoms for more than twenty-nine days. It appears that age does not influence the course of the disease; at least, differences were not evident between 6- to 10-week-old chickens and sexually mature chickens. A definite explanation can not be given for the protracted course of the disease in some birds, except that lowered vitality of the flock may aggravate the severity and duration of the symptoms.

SERUM-VIRUS NEUTRALIZATION TEST IN EMBRYONATED EGGS

An embryo-propagated strain of infectious bronchitis employed in these studies was obtained from Dr. J. P. Delaplane. This strain was originally isolated by Dr. F. R. Beaudette

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Dr. Sperling resigned August, 1949, and is now associated with the Maryland Livestock Sanitary Service, Bel Air, Md.

and had been subjected to 133 egg passages before it was received here.

At the outset, the method for propagating the virus was similar to that described by Beaudette and Hudson.[2] Later, modifications in the technique were adopted, including the use of the amnioallantoic fluid (AAF) as virus material and the employment of the allantoic chamber route for inoculation. During the early part of these investigations, the infected AAF was stored at 8 C., and later it was stored at −26 C. All inoculums were cultured for bacterial contamination. For the greater part of these investigations, eggs were obtained from infectious bronchitis-free flocks. Ten-day-old embryos were inoculated and candled daily to determine mortality. All dead embryos were examined immediately after candling or were stored at 8 C. and examined within twenty-four hours. Embryos surviving on the sixth or seventh days postinoculation were also examined. During the early part of these investigations, uninoculated control eggs were used for comparative study.

The egg-propagated virus was subjected to 351 serial passages and, for the most part, the virus killed the embryos consistently on the second day postinoculation. Gross changes in the embryos and membranes were insignificant and inconstant. The lethal titer of the virus for embryos was quite variable and seldom exceeded 10^{-4} or 10^{-5}. This virus was employed in the serum-neutralization (SN) tests in embryonated eggs.

The fact that Beach and Schalm[1] demonstrated neutralization of the virus by immune serum, using the chicken as the test animal, suggested the possibility of applying this procedure in embryonated eggs. If the latter method were found reliable in determining whether birds had been infected with the virus, it would prove a valuable means in research and diagnostic work.

Equal parts of sterile serum and undiluted AAF were combined and the mixture incubated at 37 C. for one-half hour. Embryonated eggs were inoculated with 0.2 ml. of the serum-virus mixture and incubated at 38 C. for six or seven days. Control injections (including virus and susceptible and immune serums) were incorporated into each series of tests.

In a series of tests employing the serums of 170 birds ranging in age from 22 to 104 days, it was observed that more than 91 per cent of the embryos had died by the third day postinoculation. The embryo mortality pattern was very similar to that produced by the virus when inoculated alone. Similar results (table 2) were obtained with serums from two groups of pullets, approximately 7 months old, which originated from a flock free of infectious bronchitis. The susceptibilty of the serum donors to infectious bronchitis, as determined by the neutralization method, was confirmed by the appearance of the disease in these birds when exposed later to a known infective bronchitis virus.

During the past ten years, a large number of laboratory chickens have been exposed to the infectious bronchitis virus and their serums tested for neutralizing substances. The data in table 2 are typical of results obtained with serums from birds recovered from infectious bronchitis. It is clearly evident that the

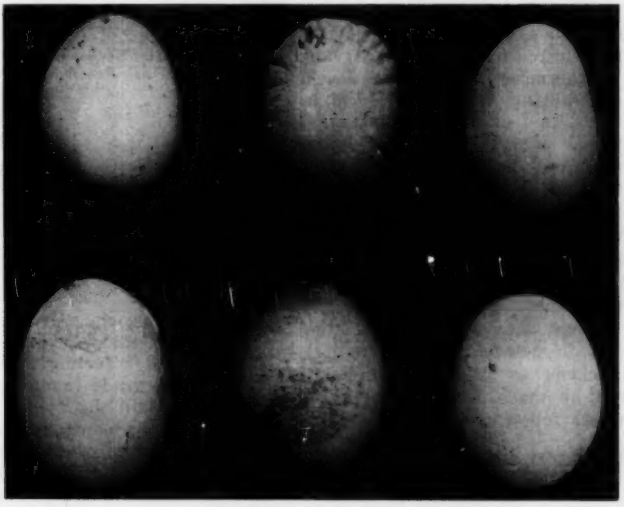

Fig. 1 — Misshapen, rough, and thin-shelled eggs produced by a flock during an outbreak of infectious bronchitis.

neutralization of the virus by the serum can be detected by inoculating the mixture into embryonated eggs.

TIME REQUIRED FOR THE DEVELOPMENT OF NEUTRALIZING SUBSTANCES

Experiments were designed to determine how soon after exposure to the virus neutralizing substances could be detected in the blood. Table 3 gives the results with 8 birds whose serums were tested before and after exposure to an infective bird-propagated virus. The serums of all birds failed to neutralize the egg-adapted virus before exposure to the bird-propagated strain. After exposure to the virus, 3 of 8 birds failed to show definite respiratory symptoms, which possibly may be explained by a decrease in the infectivity of the virus. Furthermore, the rate of development of neutralizing substances varied in different birds. In 1 case, these substances were detected as early as eleven days postinoculation and, in the remaining 7 birds, the time of appearance ranged from thirteen to thirty-six days. In some instances, there was an abrupt change from 100 per cent mortality among the test embryos to 100 per cent viability, whereas, in other cases, the transition appeared more gradual.

TABLE 1—Time of Appearance of Symptoms in Chickens Exposed to Infectious Bronchitis Virus

Age of birds (wk.)	Birds exposed (total)	No. days to first symptom and No. birds showing signs on respective days						No. symptoms
		1	2	3	4	5	6	
1- 2	4	0	4	..	..	..	..	..
3- 4	35	1	18	11	4	0	0	1
5- 6	59	11	24	18	6	..	..	..
7- 8	87	21	40	13	12	0	1	..
9-10	75	2	30	26	16	0	0	1
11-12	41	1	3	20	12	3	0	2
13-14	13	0	6	5	2	..	..	..
15-16	12	0	7	5	..	..	..	..
17-18	14	0	4	8	2	..	..	..
19-20	43	0	3	7	3	..	..	..
Over 20	24	1	1	13	5	4	..	..
Totals	377	37	140	126	62	7	1	4
Per cent		9.81	37.14	33.42	16.45	1.86	0.26	1.06

These data would suggest that the SN test to determine the immune status of chickens to infectious bronchitis should not be made for at least three to four weeks following exposure to the virus.

DURATION OF IMMUNITY

A study of the persistence of neutralizing substances in the blood of chickens recovered from infectious bronchitis revealed that, in a group of 7 birds, neutralizing substances could be demonstrated by the SN test for at least 483 days following exposure to the virus. Periodic SN tests on the serums of these birds were consistently in agreement with the results of the known immune control. Furthermore, all chickens but 1 were challenged with an infective virus and were found to be refractory to the disease. The duration of immunity among birds, recovered from either laboratory or natural infections, has not been determined. However, it is believed that the majority of birds surviving a typical outbreak of the disease enjoys an immunity for life. In some artificially infected flocks, birds which developed characteristic symptoms postinoculation have later become reinfected, as verified by the recovery of the virus. Such reinfections were usually of a mild order.

TESTING OF COMMERCIAL FLOCKS WITH THE SERUM-NEUTRALIZATION TEST

When commercial flocks had been subjected to the SN test during an active outbreak of the disease, the serums failed to neutralize the virus, and approximately 100 per cent mortality of the inoculated embryos resulted.

In one flock with a history of mild respiratory infection, 8 birds were obtained for study. The results of the SN test disclosed that serums from all but 1 bird completely neutralized the virus. The fact that the flock had manifested only a mild infection earlier, perhaps causing a low grade immunity in some birds, might explain the failure of one serum to completely neutralize the virus. However, the 8 birds, when challenged with a potent infective virus, failed to contract the disease and egg production was not affected.

TABLE 2—Serums from Susceptible Mature Birds and Their Reaction with Virus in Embryonated Eggs

Group	No. birds	Embryos inoculated	Embryo mortality by days						Mortality (%)
			1	2	3	4	5	6	
1	16	80	0	63	14	2	1	..	100.00
2	23	115	2	90	19	0	1	3*	97.39
Totals	39	195	2	153	33	2	2	3*	98.46

*Live embryos.

During the past eight years, commercial flocks have been tested by the SN test and the bird-virus challenge method as a means of determining their immunity to infectious bronchitis. At the outset, only flocks with a respiratory history suggestive of infectious bronchitis were selected but, later, other types of flocks were also included. A flock sample of 4 birds was submitted to the laboratory. The birds were bled for the SN test and inoculated intratracheally with an infective bronchitis virus. After a five- to seven-day observation period for symptoms, the birds were killed and examined for lesions.

In table 4 are given the composite results for 297 commercial flocks (281 immune and 16 susceptible) representing 1,188 serum samples tested during three calendar years. The results for the remaining years were quite similar and are not included in this paper. With the immune group serums, the embryo mortality was quite consistent with a peak on the

TABLE 3—Reaction of Serums from Chickens Before and After Exposure to Bronchitis Virus

Bird (No.)	Date hatched	SN tests in embryonated eggs: Date	Dead‡ (total)	Mortality by days: 1	2	3	4	5	6	Exposure to virus: Date	Symptoms	Neutralizing substance detected after exposure (days)
4201	12/12/41	3/12/42*	7/7	0	5	2	..	..	..			
		3/16	7/7	0	7	..	..	..	..			
		3/19	5/5	0	4	0	1	..	..			
										3/20/42	+	
		3/26	4/4	0	2	0	2	..	..			
		3/30	3/5	0	0	2	1	0	0			
		4/6	0/5	0	0	0	0	0	0			17
910	2/ 2/42	3/30	5/5	0	4	1	..	..	..			
										4/3	+	
		4/9	1/5	0	1	0	0	0	0			
		4/17	4/5	0	0	2	2	0	0			
		4/23	3/5	0	0	2	0	1	0			
		4/30	0/5	0	0	0	0	0	0			27
914	2/ 2/42	4/6	5/5	0	5	..	..	..	..			
										4/8	+	
		4/17	5/5	0	2	2	1	..	..			
		4/23	4/5	0	4	0	0	0	0			
		4/30	5/5	0	0	4	1	..	..			
		5/7	2/5	0	0	2	1	0	0			
		5/14	0/4	0	0	0	0	0	0			36
956	2/ 2/42	4/6	5/5	0	5	..	..	..	..			
										4/8	+	
		4/17	1/5	0	0	0	1	0	0			
		4/30	0/5	0	0	0	0	0	0			11
		5/7	0/5	0	0	0	0	0	0			
930	2/ 2/42	4/6	5/5	0	4	1	..	..	..	4/17	—	
		4/23	5/5	0	0	5	..	..	..			20
		5/7	1/5	0	0	0	0	0	1			
		5/14	0/4	0	0	0	0	0	0			
		7/6	1/5	0	0	1	0	0	0			
967	2/ 2/42	4/6	5/5	0	3	2	..	..	..			
										4/17	—	
		4/23	5/5	0	2	2	9	1	..			
		4/30	1/5	0	0	0	1	0	0			13
		5/7	1/5	0	0	0	0	1	0			
		5/14	0/4	0	0	0	0	0	0			
963	2/ 2/42	4/6	5/5	0	3	2	..	..	..			
										4/17	+	
		4/23	5/5	0	4	1	..	..	..			
		4/30	5/5	0	0	2	2	1	..			
		5/7	3/5	0	0	3	0	0	0			
		5/14	1/4	0	0	0	0	0	1			27
		7/6	0/5	0	0	0	0	0	0			
949	2/ 2/42	4/17	5/5	0	4	0	1	..	..			
										4/17	—	
		4/23	5/5	0	4	1	..	..	..			
		4/30	5/5	0	2	2	1	..	..			
		5/7	5/5	0	1	0	0	0	4			
		5/14	1/4	0	0	1	0	0	0			27
		7/6	1/5	0	0	1	0	0	0			

*Controls (virus, susceptible serum, and immune serum) conducted with all tests were satisfactory. ‡Numerator = number of dead embryos; denominator = number of embryonated eggs inoculated.

third day following inoculation. However, the percentage of embryo mortality was well within limits which indicate that the donors of the serums had been exposed to infectious bronchitis virus. In contrast, the serums from the susceptible birds failed to neutralize the virus in 93.75 per cent of the embryos. The controls (including virus and nonimmune and immune serums) tested simultaneously with the unknown serums gave satisfactory results.

TABLE 4—Composite Results of Serum-Neutralization Tests for Infectious Bronchitis Virus plus Serums Collected from Commercial Flocks

Year	No. flocks	No. serums	No. embryos inoculated	Daily embryo mortality 1	2	3	4	5	6	Total mortality No.	%
1942	44	176	879	3	45	104	89	39	23	303	34.47
1943	100	400	1,995	38	64	123	83	85	57	450	22.56
1948 Immune	137	548	2,192	38	102	111	69	58	50	428	19.52
1948 Susceptible	16	64	256	2	77	78	46	24	13	240	93.75

Among 645 flock consignments tested by the SN method during the past eight years, all of which were identified as immune to infectious bronchitis, the serums from 598 flocks (92.4%) neutralized the virus in more than 50 per cent of the embryos inoculated, as revealed by embryo mortality. While this mortality may be regarded as excessive for a well-defined test result, the data, when compared with that of the virus and serum controls, reveal that the flocks had been exposed to infectious bronchitis.

Serums from some flocks gave variable results. To illustrate, two serums among a flock sample of 4 birds failed to neutralize the virus, and all embryos died by the sixth day postinoculation. In the case of the other two serums, 80 and 100 per cent, respectively, of the embryos survived. As another illustration, an embryo mortality of 60 per cent or greater occurred with the different serums. The mortality pattern approached that of a susceptible serum. Retests of the same serums yielded essentially the same results. It is apparent that in some flocks recovered from infectious bronchitis, serums of certain birds may not completely neutralize an egg-adapted virus, as demonstrated by the embryo-inoculation method.

In correlating the results of the SN test with the symptoms and lesions obtained in birds inoculated with an infective virus, signs were observed infrequently in birds whose serums failed to protect 50 per cent of the inoculated embryos (table 5). These findings are in direct contrast to what may be expected with immune serums. Among the 645 flock consignments tested by the bird-inoculation method, 24 flocks exhibited some degree of respiratory symptoms but, in 22 of the 24 flocks, only 1 bird per flock sample manifested symptoms. On the other hand, birds from 228 flocks exhibited lesions following virus challenge and, in over 50 per cent of the consignments, only 1 bird manifested lesions. In many instances, the extent of the respiratory lesions was extremely limited. The proportion of birds exhibiting lesions was greater among the flock samples whose serums protected more than 50 per cent of the inoculated embryos against the virus. The immunity of these birds was ascertained more definitely through the field inoculation of the young stock when the disease did not spread to the hens from which samples had been tested for immunity.

A satisfactory explanation can not be offered at this time as to why birds of flocks recovered from infectious bronchitis may exhibit a partial syndrome of the disease upon inoculation with the virus. That local respiratory tissue immunity was inadequate to afford complete protection against the invasion of the infective virus, and yet that sufficient circulating antibodies existed in the serums to neutralize the virus in the embryos is a possibility that might be considered.

According to these results, the SN test was not as reliable as the bird-inoculation method in determining the immune status regarding infectious bronchitis. However, more recently, Jungherr[4] and Fabricant[3] have reported that the SN test is very reliable when a high titer, egg-adapted virus (10^{-4} to 10^{-6}) is employed and when certain technical details are observed. This

being the case, the SN test using the embryo method and the bird-inoculation method with an infective virus may be useful procedures in evaluating flock immunity for infectious bronchitis.

INFLUENCE OF INFECTIOUS BRONCHITIS ON EGG PRODUCTION

In Massachusetts, the greatest loss from infectious bronchitis occurs in mature flocks which are in egg production. A rapid decline in production may occur together with a marked alteration in the exterior and interior quality of the egg, such as misshapen appearance, rough and thin shell, bleached color, and watery albumen (fig. 1). Recovery to preinfection levels in egg production occurs rarely and, in many instances, the abnormal shell quality of the egg may continue to appear as long as the flock is maintained. Egg records of numerous affected flocks have been studied and a typical case may be cited. The production declined from 70.0 to 2.4 per cent within two weeks following the appearance of the disease. The level of production remained low for approximately four weeks and, at nine weeks after the appearance of the disease, egg production had increased to approximately 50 per cent. Using egg prices which were current at the time of the outbreak, it was calculated that the flockowner suffered a loss of approximately $1 per bird during a nine-week period. Numerous laying flocks in Massachusetts have been liquidated as the result of infectious bronchitis outbreaks. The losses from this disease among laying flocks exceed those suffered from Newcastle disease.

IMMUNIZATION OF COMMERCIAL FLOCKS

Recognizing that healthy young birds can be successfully immunized by exposure to infectious bronchitis virus with little or no retardation in growth, this immunization procedure was applied to commercial flocks on premises where the disease had been identified.[6] The purpose of this program was to immunize the growing stock so that the flock would be protected against the disease during the laying period.

The procedure of the program was briefly as follows.

At the outset, only flocks on premises where the disease had occurred were enrolled in the program. It was recommended that the young birds be inoculated not prior to 8 weeks nor after 16 weeks of age. However, in some instances, these age limits could not be observed. The most desirable age for exposure was 10 to 14 weeks. Approximately, 2 to 5 per cent of the birds in a flock were intratracheally inoculated (swab method) by trained inoculators using a bird-propagated virus. After the inoculated birds developed the disease, they, in turn, spread the infection to the uninoculated birds in the flock. If symptoms did not appear in seven to ten

TABLE 5—Correlation of Embryo Mortality with Bird Virus Challenge Results Among Bird Consignments from Flocks Submitted for Immunity Tests

Year	Embryo mortality (%)	No. flocks	Symptoms					Lesions				
				Birds per flock					Birds per flock			
			Flocks	1	2	3	4	Flocks	1	2	3	4
1942	<50	31	1	1	..	..	..	12	9	3	0	1
	>50	9	0	..	..	..	..	2	1	1	..	..
1943	<50	98	2	2	..	..	..	33	13	12	8	..
	>50	6	0	..	..	..	..	2	1	1	..	..
1944	<50	70	3	3	..	..	..	16	10	3	3	..
	>50	3	0	..	..	..	..	0	..	..	..	..
1945	<50	12	0	..	..	..	..	2	2	..	..	..
	>50	1	0	..	..	..	..	0	..	..	..	..
1946	<50	24	1	0	1	..	..	9	6	2	1	..
	>50	6	0	..	..	..	..	1	1	..	..	..
1947	<50	49	2	2	..	..	..	6	3	2	1	..
	>50	10	1	1	..	..	..	3	2	0	1	..
1948	<50	163	8	7	1	..	..	57	32	13	7	5
	>50	7	1	1	..	..	..	6	4	1	1	..
1949	<50	151	5	5	..	..	..	74	35	23	10	6
	>50	5	0	..	..	..	..	4	3	0	1	..
Totals	<50	598	22	20	2	..	..	210	110	58	30	12
	>50	47	2	2	..	..	..	18	12	3	3	..
Grand total		645	24	22	2	..	..	228	122	61	33	12

> = greater than; < = less than.

days following inoculation, the flock was reinoculated.

During the past nine years, 2,541 flocks were enrolled in the infectious bronchitis control program. In the majority of inoculated flocks, the results were satisfactory. The incubation period varied from two to eight days. The severity and duration of symptoms also varied in different flocks or among groups of birds within the same flock. In the majority of flocks, the symptoms had disappeared within ten to twelve days. However, in some cases, symptoms were evident for twenty-eight days or even longer. A definite explanation is not offered for the variable results obtained in some flocks. It may be that one of several or a combination of factors—such as time and degree of exposure to the virus, loss in infectivity of the virus prior to inoculation, environmental conditions on the premises, inherent resistance to the disease, age, and the actual health status of the flock—may be responsible. A decrease in feed consumption of 50 per cent was frequently observed in flocks. Excessive mortality resulted infrequently and usually could be attributed to complications from other causes.

In a few instances, the mature stock, supposedly immune, contracted the disease from the inoculated young stock. Some of these breaks were definitely confirmed by virus isolation.

While some flocks did not develop sufficient immunity, the majority of inoculated flocks appeared to be adequately protected against natural or artificial exposure to the virus. The protection against the disease has saved the Massachusetts poultrymen thousands of dollars and has helped many flockowners to continue their operations from year to year uninterrupted by outbreaks of infectious bronchitis.

SUMMARY

The results of investigations concerning infectious bronchitis are recorded and discussed.

It was found that the disease is highly contagious and spreads with great rapidity. The incubation period may vary from twenty-four hours to six days. The rapid spread of the disease presents a problem in establishing barriers against the transmission of the infection within a flock. Practical preventive measures instituted against the spread of the disease usually fail.

The course of the disease is relatively short, the majority of birds exhibiting symptoms from one to eleven days after exposure. In some instances, the disease may follow a protracted course.

Employing an egg-adapted virus, it was demonstrated by means of the serum-neutralization (SN) test that serums from birds recovered from the disease were capable of neutralizing the virus. This test and the method of bird inoculation with an infective virus are useful procedures in the diagnosis of the disease among recovered flocks. However, the reliability of these methods is dependent upon the proper handling of the egg-adapted virus, in the case of the SN test, plus exacting technique. A highly infective virus is essential in the case of bird inoculation.

Losses from the disease in laying flocks may approximate $1 per bird, as the result of decreased egg production and abnormal egg quality.

A program of artificial exposure of young birds to an infective virus has enabled flockowners to immunize their flocks, thus affording protection against the disease during the productive period of the flock. In an occasional flock, insufficient immunity is developed to prevent a natural outbreak. However, this control program has greatly benefited the poultrymen in spite of its shortcomings. Further investigation is urgent to improve methods of coping with this disease.

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Effect of a Vitamin B_{12} Concentrate and Liver Meal on the Hematology of Chicks Fed an All-Plant Protein Ration

M. J. SWENSON, D.V.M., M.S., Ph.D.

Ames, Iowa

IN A PREVIOUS paper,[1] the effect of a vitamin B_{12} concentrate and liver meal on growth and on feed efficiency of chicks fed an all-plant protein ration was reported. Significant growth responses were obtained in the female chicks by replacing 3 per cent of the soybean meal in the basal ration with 3 per cent liver meal, and also by replacing 0.5 per cent of the soybean meal in the basal ration with 0.5 per cent animal protein factor (APF) supplement No. 3 of Merck and Company. The APF ration contained 62.5 μg. of vitamin B_{12} per pound by the *Lactobacillus lactis* Dorner method of assay. Under the same conditions, growth responses of the male chicks were not significant statistically except at 2 weeks of age. Environmental effects* were present in the weight gains of the male chicks and the standard errors of the mean weight gains of the male chicks were larger than those of the female chicks. These findings indicate that female chicks should be a better assay animal for vitamin B_{12} than male chicks. The feed efficiency of the chicks fed the liver meal ration was the best throughout the experiment. There was no significant difference in feed efficiency among the chicks fed the basal and APF rations.

A study of various blood constituents in these groups of chicks is reported herein. Hemoglobin, hematocrit, sedimentation rate, and counts of erythrocytes, total leucocytes, and differential leucocytes have been determined. A later publication will include the observed histopathologic changes in selected chick tissues.

Materials and Methods

Ninety New Hampshire chicks, hatched from eggs produced by hens fed an all-plant protein ration for five months, were assigned at random to nine groups with 10 chicks in each group. A description of the rations and experimental procedure has been described in a previous paper.[1]

The blood studies were begun when the chicks were 4 weeks of age. Six chicks were bled every other day, with different chicks being bled each time. The bleeding was continued in this manner until the 90 chicks were bled. At this time, the first 4 chicks of each group, that had been bled previously, were bled again to determine if age produced a significant change in the blood constituents. By the method described by Hofstad,[2] 2 ml. of blood were obtained directly from the heart. Each blood sample was placed in a test tube containing 2.4 mg. of dry ammonium oxalate and 1.6 mg. of dry potassium oxalate. This anticoagulant is described by Heller and Paul[3] and by Wintrobe.[4]

The hemoglobin content of the chick blood was determined with a Klett-Summerson photoelectric colorimeter. A stock standard acid hematin solution was prepared after the iron content of a sample of chicken blood (30 ml.) was determined by the method described by Wong.[5] The blood was then diluted with 0.1 normal HCl in a 100-ml. volumetric flask, so that the stock standard acid hematin solution contained 3 per cent hemoglobin.

Schultze and Elvehjem[6] overcame the turbidity of acid hematin solutions of chicken blood by first diluting the blood with 0.4 per cent NH_4OH, as Palmer[7] did with human blood, and then adding a few drops of concentrated HCl. The unknown blood sample and the standard acid hematin solution were treated in this manner. Numerous checks were made on the standard acid hematin solution by using NH_4OH and then acidifying with concentrated HCl and by using 0.1 normal HCl without NH_4OH. In all cases, the readings

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The author is now at the School of Veterinary Medicine, Kansas State College, Manhattan.

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*Test of significance performed in the analysis of variance of the weight gains by dividing the mean square for pens treated alike by the mean square for individuals.

were lower on the colorimeter when NH_4OH was used. The standard acid hematin solution was prepared fresh each week by diluting 2.5 ml. of the stock standard to 100 ml. with 0.4 per cent NH_4OH and concentrated HCl. NH_4OH (0.4%) was added to the 2.5 ml. of stock standard in a 100-ml. volumetric flask, leaving approximately 5-ml. space for the HCl and adjustment of the pH to 1.1. This pH was obtained when the method of Schultze and Elvehjem[8] was followed. The following is a summary of the method used in determining the hemoglobin content of chicken blood:

Place 20 ml. of 0.4 per cent NH_4OH in a 50-ml. test tube.
Add 0.1 ml. of blood with blood pipette, rinsing three times with the NH_4OH solution.
Stopper test tubes and mix well by inverting three times.
Let stand at least one minute.
Add 0.48 ml. of concentrated HCl, or until pH is 1.1.
Stopper test tubes and mix well by inverting three times.
Let stand forty minutes.
Read unknown and known standard acid hematin solutions in colorimeter.

Calculation

$$\frac{\text{Reading of unknown} \times 0.075 \times 100 \times 20.58}{\text{Reading of standard} \quad 0.1 \quad 100} =$$

grams of hemoglobin per 100 ml. of blood.

The 0.075 is the grams per 100 ml. of hemoglobin in the acid hematin standard,[8] and the 0.1 is the amount of blood used expressed in milliliters. The 20.58 represents the number of milliliters of diluted blood.

The erythrocyte and leucocyte counts were made by Wiseman's method, as described by Olson.[9, 10] Olson found that Wiseman's method was the most satisfactory of the five methods studied for counting erythrocytes. This method was also the better of the two indirect methods for counting leucocytes. In counting the stained erythrocytes and acidophilic granulocytes, both counting chambers of a hemacytometer were used. The counts were made on both sides and averaged.

A differential leucocyte count is necessary in arriving at a total leucocyte count when using Wiseman's indirect method. In calculating the total leucocyte count, the formula as presented by Olson[10] was used. Differential leucocyte counts were made by staining the blood films with a May-Grünwald and Giemsa combination stain. In making the differential leucocyte counts, 200 cells were counted by the "four-field meander" method described by Gradwohl.[11]

The sedimentation rate of the erythrocytes was determined at intervals of one-half, one, two, three, and six hours after the Wintrobe hematocrit tubes were filled. The tubes were filled with blood by using Wintrobe-filling pipettes. The tubes were held in a vertical position in a blood sedimentation rack designed for Wintrobe tubes. At the end of the six-hour period, the hematocrit tubes were centrifuged for thirty minutes at 3,000 revolutions per minute. The volumes of packed erythrocytes, packed leucocytes and thrombocytes, and plasma expressed in per cent were recorded.

RESULTS

Table 1 presents the mean hemoglobin values of the 90 male and female chicks fed the three rations. Analysis of variance of these data does not show that the treatments caused a significant difference[12] in the hemoglobin values. However, in checking the least significant differences among the hemoglobin values of male and female chicks fed the basal, liver meal, and APF rations, the hemoglobin mean for female chicks fed the basal ration (6.47) is significantly different from the mean of those fed the liver meal ration (7.01).

The mean and standard error of the mean for the hemoglobin values of chicks for age 1 are 6.66 ± 0.06 and for age 2, 7.09 ± 0.06. These means were obtained from the hemoglobin values of the first 4 chicks in each pen (age 1) and the second hemoglobin values (age 2) of the same chicks determined twenty-one to thirty-seven days later. Statistically, there is a highly significant difference (1% level) between the

TABLE 1—Hemoglobin Values and Erythrocyte Counts

	Ration		
	Basal	Liver Meal	APF
Sex	Mean Error*	Mean Error*	Mean Error*
Male	7.02 ± 0.12	6.68 ± 0.14	6.76 ± 0.12
Female	6.47 ± 0.22	7.01 ± 0.19	6.70 ± 0.23
	Erythrocytes per cubic millimeter of blood 10^6		
Male	2.519688 ± 0.043269	2.651667 ± 0.049962	2.589412 ± 0.041977
Female	2.583636 ± 0.082240	2.646111 ± 0.072529	2.540385 ± 0.085344

*Standard error of the mean.

hemoglobin values obtained from the chicks of two different ages.

Erythrocyte counts and the other blood determinations were made on the same blood samples on which the hemoglobin was run. Table 1 shows the mean erythrocyte counts of the chicks fed the three rations. Analysis of variance of the erythrocyte counts shows that significant treatment effects were not present. The differences between the means were less than the least significant differences. The mean and standard error of the mean of the erythrocyte counts of chicks per cubic millimeter of blood for age 1 were 2,543,194 ± 41,167 and for age 2 were 2,541,944 ± 41,167. No significant difference existed between the erythrocyte counts of the two ages of chicks.

Table 3 gives the mean sedimentation rates of the male and female chicks fed the three rations. Analyses of variance show no evidence of treatment effects in the sedimentation rates. Environmental effects were present in the sedimentation rates of erythrocytes of male chicks at one-half hour and at two hours for female chicks. The differences between the means (table 3) in each row were less than the least significant differences. There is no significant difference between the sedimentation rates of erythrocytes from the two ages of chicks. Therefore, the treatments did not cause a significant change in the sedimentation rates with age.

TABLE 2—Total Leucocyte and Differential Counts per Cubic Millimeter of Chick Blood

		Ration					
		Basal		Liver Meal		APF	
Total	Sex	Mean	Error*	Mean	Error*	Mean	Error*
Leucocytes	M	25,959.0	± 1,893.3	27,852.5	± 2,186.1	27,837.2	± 1,836.7
Leucocytes	F	31,528.9	± 4,155.8	30,672.7	± 3,665.0	26,206.5	± 4,312.6
Lymphocytes	M	16,734.1	± 1,620.5	17,199.3	± 1,871.2	19,003.0	± 1,572.1
Lymphocytes	F	22,388.0	± 4,036.7	21,469.5	± 3,560.1	18,298.7	± 4,189.1
Heterophils	M	7,281.9	± 997.4	8,402.8	± 1,151.7	6,752.7	± 967.6
Heterophils	F	6,707.4	± 533.5	6,887.0	± 470.5	5,820.3	± 553.6
Monocytes	M	1,587.6	± 177.7	1,920.5	± 205.2	1,751.7	± 172.4
Monocytes	F	1,895.2	± 187.9	1,894.6	± 165.7	1,707.1	± 195.0
Eosinophils	M	259.7	± 35.5	245.4	± 41.0	201.5	± 34.5
Eosinophils	F	292.6	± 72.9	288.9	± 64.3	213.8	± 75.7
Basophils	M	95.7	± 55.0	84.6	± 63.5	128.3	± 53.4
Basophils	F	245.8	± 54.1	132.7	± 47.7	166.6	± 56.1

*Standard error of the mean.

The mean total and differential leucocyte counts are presented in table 2. Analyses of variance of the different counts per cubic millimeter of blood from male and female chicks fed the three rations reveal the absence of significant treatment effects, whereas environmental effects were present in the total leucocyte and lymphocyte counts of female chicks at the 5 per cent level of significance and also in the basophil counts of male chicks at the 1 per cent level. The differences between the treatment means were less than the least significant differences. Therefore, no significant difference exists among the three treatment means in each row of table 2. Statistically, the number of eosinophils per cubic millimeter of blood was the only group of blood cells to change significantly with age. The difference in the eosinophil counts between age 1 and age 2 was significant at the 5 per cent level but not at the 1 per cent level.

The mean hematocrit readings expressed in per cent are given in table 4. The analyses of variance demonstrate that treatments did not produce a significant change in the hematocrit readings. The differences between the means of the hematocrit readings of the three rations (table 4) were less than the least significant differences. However, it was observed that the difference (1.79) and the least significant difference (1.90) for packed erythrocytes of female chicks fed the basal and liver meal rations were very close. It was found that this difference (1.79) was equal to the least significant difference when a t-value between 6 and 7 per cent was used instead of the usual 5 per cent value. Age did not produce a significant difference in the hematocrit readings.

DISCUSSION

The method used in determining hemoglobin corrected the higher readings usually obtained from the nucleated erythrocytes of chicken blood. The method described by Schultze and Elvehjem[6] was employed with

certain modifications enabling the use of the Klett-Summerson photoelectric colorimeter. The significant difference observed between the hemoglobin levels of female chicks fed the basal and liver meal rations (table 1) was also found to be reflected in the packed erythrocyte volume (table 4).

TABLE 3—Sedimentation Rates of Chick Erythrocytes Expressed in Millimeters

		Ration		
		Basal	Liver Meal	APF
Time	Sex	Mean Error*	Mean Error*	Mean Error*
0.5 hr.	M	0.81 ± 0.26	0.46 ± 0.30	0.65 ± 0.25
0.5 hr.	F	0.50 ± 0.12	0.44 ± 0.11	0.38 ± 0.13
1 hr.	M	2.13 ± 0.50	1.33 ± 0.57	1.79 ± 0.48
1 hr.	F	1.25 ± 0.20	1.36 ± 0.18	1.31 ± 0.21
2 hr.	M	4.75 ± 0.71	3.08 ± 0.82	4.24 ± 0.70
2 hr.	F	3.68 ± 0.63	3.28 ± 0.56	3.38 ± 0.66
3 hr.	M	8.63 ± 1.11	5.50 ± 1.29	7.35 ± 1.08
3 hr.	F	6.89 ± 1.02	6.17 ± 0.90	5.85 ± 1.12
6 hr.	M	16.81 ± 1.72	12.50 ± 1.99	15.38 ± 1.67
6 hr.	F	15.43 ± 1.91	13.78 ± 1.68	12.62 ± 1.98

*Standard error of the mean.

It is advisable to calculate the least significant difference between two means when more than two treatments have been used. There are times, as shown in the hemoglobin values, when a significant mean difference exists while the analysis of variance shows no treatment effects. This method leads to the same conclusions as those obtained from individual comparisons.

Wiseman's method of counting erythrocytes and leucocytes was used, because Olson[9] found this method to be the most satisfactory for counting erythrocytes. Since vitamin B_{12} is known to have an erythropoietic function, the method most likely to detect smaller differences in the erythrocyte counts was chosen.

TABLE 4—Hematocrit Readings of Chick Blood Expressed in Per Cent

		Ration		
		Basal	Liver Meal	APF
	Sex	Mean Error*	Mean Error*	Mean Error*
Erythrocytes	M	30.63 ± 0.49	30.33 ± 0.57	30.91 ± 0.48
Erythrocytes	F	29.79 ± 0.79	31.58 ± 0.70	30.15 ± 0.82
Leucocytes and Thrombocytes	M	1.53 ± 0.19	2.08 ± 0.21	1.47 ± 0.18
Leucocytes and Thrombocytes	F	1.75 ± 0.28	1.67 ± 0.25	2.12 ± 0.30
Plasma	M	67.84 ± 0.37	67.58 ± 0.43	67.62 ± 0.36
Plasma	F	68.46 ± 0.84	66.75 ± 0.74	67.73 ± 0.87

*Standard error of the mean.

Corrections in the sedimentation rates of erythrocytes according to volumes of packed erythrocytes were not made. Apparently, a chart correcting the sedimentation rate of chicken erythrocytes according to volume of packed erythrocytes is not available. The chart used in correcting the sedimentation rate of human erythrocytes[4] may be applicable to other species of mammalian blood; however, it was not considered practical for chicken blood without proof by further investigation.

The measurement of packed leucocytes and thrombocytes in a hematocrit tube is not accurate to determine total white cell counts because readings of 1 mm. or 0.5 mm. do not bring out the small differences in blood samples necessary for accuracy and precision. The error in reading is extremely high. In this investigation, the readings for packed leucocytes and thrombocytes were recorded in order to obtain more accurate readings for plasma and packed erythrocyte volumes. The white cell counts made by using a hemacytometer and stained blood films are more reliable.

Summary and Conclusions

1) Statistically, a significant difference (5% level) was found between the hemoglobin levels of the female chicks fed the basal and liver meal rations, while no significant difference existed between the hemoglobin levels of chicks fed the basal and animal protein factor (APF) rations and also between those fed the liver meal and APF rations.

2) The hemoglobin levels of the male chicks fed the three rations were not significantly different.

3) The packed erythrocyte volume of female chicks fed the basal and liver meal rations approached the 5 per cent level of significance.

4) Treatment effects on erythrocyte counts, total leucocyte counts, differential counts, sedimentation rates, plasma volume, and packed leucocytes and thrombocytes were not significant.

5) Environmental effects produced a highly significant difference (1% level) in the basophil counts of male chicks, a significant difference in the sedimentation rate of erythrocytes of male chicks in one-half hour and, in female chicks, a significant differ-

ference in the eosinophil counts, whereas the remaining blood determinations had not changed significantly.

ence in the total leucocyte counts, lymphocyte counts, and sedimentation rate of erythrocytes in two hours.

6) Age (21 to 37 days) produced a highly significant difference in the hemoglobin levels of chicks and a significant dif-

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Influence of Environmental Factors on Viability of Newcastle Disease Virus

OLGA M. OLESIUK, B.A., M.S.

Amherst, Massachusetts

THE ECONOMIC significance of Newcastle disease (ND) is recognized and has attracted the interest of many investigators. However, only limited studies have been made on the actual behavior of the virus. Some problems of transmission might be clarified by thorough study of certain properties of the virus.

The purpose of this investigation was to determine the length of time the virus would remain viable on sterilized, different materials when subjected to storage under various environmental conditions.

The duration of virulency and viability of Newcastle disease virus (NDV) has been studied, in part, by various workers. The studies on survival of both foreign and American types of virus have been summarized by Beaudette.[2-4] Some of the more outstanding viability and virulency results are cited in this paper.

Doyle[7] found the virus active for forty-eight hours at 37 C. but inactive after seventy-two hours. Farinas[8] found that virus-infected crop contents in a liquid state, stored in cotton-stoppered test tubes, remained virulent for fifteen days at room temperature; but, if allowed to dry at room temperature on absorbent cotton, the virus was inactivated in five days. Asplin[1] reported that virus on filter paper and eggshells, and in sterile feces on jute bags, all held in dry heat at 36.7 C., was still active at six, twenty-four, and seventy-two hours, but was inactive at twelve, forty-four, and ninety hours. Jungherr[12] reported that, on sterile strips of burlap, the virus was viable for fifty-five days at 22 C. (humidity 7%). On nonsterile burlap, the virus was viable for twenty-two days at the following temperatures: –1.7 C. (humidity 45%), 16 C. (humidity 51%), and 22 C. (humidity 7%).

The survival period of the virus was of longer duration if the virus-contaminated materials were stored at icebox or refrigerator temperatures. Doyle[7] found that virus in a 50 per cent solution of glycerin in normal saline was active after 197 days but not after 259 days. Farinas[8] reported that virus in liquid crop contents suspended in distilled water and stored in cotton-stoppered flasks remained virulent for five months and eleven days. Hudson[10] observed that virus-infected brain and spleen suspended in 50 per cent glycerin and held at 1 C. for 239 days produced death in birds after laryngeal inoculation. Iyer[11] found that pulp of infected organs, such as the liver and spleen, dried *in vacuo* over phosphorus pentoxide and stored in the refrigerator, was virulent for about three years. Brandly, Moses, Jungherr, and Jones[5] observed that amnioallantoic fluid stored in a moist or lyophilized state at 0 to –72 C. was of unaltered infectivity after twenty-three months.

MATERIALS AND METHODS

This investigation was divided into two trials; trial 1 extended over a period of eighteen months, while trial 2 continued for nine months. In trial 1, the test materials were stored at five different conditions of temperature: incubator (37 C.), normal room (20 to 30 C.), hen house (–11 to 36 C.), refrigerator (3 to 6 C.), and deep freeze (–26 C.). In trial 2, the materials were stored at incubator and normal room temperatures.

The maximum period of viability of the virus on the materials listed was determined. All test materials, with the exception of fresh eggs and whole blood, were sterilized in the autoclave at 15-lb. pressure for twenty minutes. Undiluted virus suspension was added to the sterile material, the amount depending upon the nature of the material.

The first viability study was started on Oct. 30, 1948, and the final tests reported in this study were completed on April 21, 1950. The second viability study ran from Feb. 9, 1949, to Nov. 2, 1949.

Virus Strain.—The strain of virus (9251) used in this study was isolated in this laboratory in the latter part of 1945 from a flock experiencing a natural disease outbreak. Since that time, the virus has been passed through chicken embryos 87 times. The virus originally isolated had a lethal titer of 10^{-8} for chicken embryos.

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The virus used to seed the various test materials in this study was produced in 9- to 11-day-old embryos, using the allantoic cavity route of inoculation. All eggs used in this study were obtained from hens susceptible to ND. The amnioallantoic fluid was stored at −26 C., until an amount sufficient to seed all the test materials was collected. The lethal titer of the virus for chicken embryos was 10^{-8}.

Seeding of Test Materials.—Strips of filter paper, burlap, and cotton cloth (½ by 2 in.) were seeded with virus and stored in cotton-stoppered tubes (⅜ by 3 in.). The filter paper was moistened with 0.2 ml. of virus, the burlap with 0.5 ml. of virus, and the cloth strip with 0.2 ml. of a 1:10 virus-blood dilution.

A 1:10 virus-broth mixture (pH 7.2) and a 1:10 virus-saline mixture (pH 7.8) were used in this study. Samples sufficient for one test were prepared for storage in the hen house and deep freeze. An additional variable (freezing and thawing) would have been introduced if individual samples had not been prepared for these two conditions of storage. The materials stored at the other conditions were in volumes sufficient for two to five tests. These two test materials were stored in cotton-stoppered test tubes and rubber-stoppered vials.

The undiluted amnioallantoic fluid containing the virus was also subjected to storage under the various environmental conditions. Individual samples were prepared for storage in the hen house and deep freeze. The amnioallantoic fluid was tested for viability and, in addition, was titrated in embryos. Hemagglutination and hemagglutination-inhibition tests were also made prior to embryo passage at each condition of storage.

Mash, fecal matter, eggshells, and soil were prepared in individual samples as follows: 1 Gm. of chicken mash (pH 5.7); 1 Gm. of dried chicken feces (pH 6.3); 2 Gm. of eggshells; and 2 Gm. of soil (pH 5.2). To each material was added 1 ml. of virus. The eggshells were stored in cork- and cotton-stoppered tubes (¾ by 4 in.), the soil in rubber- and screw-topped vials, and the mash and feces in rubber-stoppered vials.

Small amounts of chick down were packed loosely into rubber-stoppered vials and moistened with 0.3 ml. of virus.

Fresh infertile eggs were inoculated with 0.2 ml. of virus.

In trial 2 of this viability study, the test materials were prepared in a similar manner, except for the modifications in the pH values of some of the materials which were as follows: broth, pH 7.0; saline, pH 7.1; soil, pH 4.9; feces, pH 6.8; and mash, pH 5.5. The lethal titer of the virus was 10^{-9} for chicken embryos.

Demonstrating the Virus.—Test materials, except broth, saline, amnioallantoic fluid, and fresh eggs, were washed with broth in sterile containers, the quantity of broth depending upon the test material, but not more than 5.0 ml. in any case. The materials were allowed to stand, with frequent agitation, for one hour at room temperature prior to embyro inoculation. The broth and saline were inoculated into embryos without further preparation. In the case of whole eggs, yolk or yolk diluted with broth was used for embryo inoculation. The amnioallantoic fluid containing the virus was diluted with broth when the embryo lethal titer of the virus was to be determined. The standard dose for inoculation was 0.2 ml. into each of 4, 9- to 11-day-old embryos for each test material. If several consecutive negative tests were obtained, two or three samples of each test material, except broth, saline, and amnioallantoic fluid, were pooled, washed, and then inoculated into embryos.

Several criteria were adopted for establishing presence of the virus. A positive test was determined by 75 to 100 per cent embryo mortality at either the first or second passage of the recovered virus. Even though 75 per cent mortality may have been obtained on first embryo passage, second passages were made in most cases to establish definitely virus pres-

TABLE 1—Duration of Viability of Newcastle Disease Virus

Test material	Incubator 37 C. (Days)		Normal room 20 to 30 C. (Days)		Hen house −11 to 36 C. (Days)	Refrigerator 3 to 6 C. (Days)	Deep freeze −26 C. (Days)
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 1	Trial 1
Burlap	*	14	16	9	108	123	538**
Filter paper	*	28	25	49	129	157	538
Cloth	10	16	52	35	145	193	538
Broth	46	14	192	112	228	538	538
Saline	17	35	87	71	228	451	538
Eggshells	*	7	52	35	228	258	538
Mash	52	56	66	56	172	258	538
Chicken feces	25	56	94	71	172	538	538
Down	87	28	192	134	255	538	538
Soil	25	2	60	71	172	235	538
Eggs	94	126	235	197	255	538	...
A.A.F.***	31	35	66	94	199	258	538

*The end point of viability was less than ten days. **The duration of the study was 538 days. ***Undiluted amnioallantoic fluid.

ence. Mortality occurring on the first day after inoculation was not considered significant. If the recovered virus suspension were contaminated with bacteria, it was treated with penicillin and streptomycin at the rate of 10,000 units and 0.025 gr., respectively, per milliliter of suspension. The suspension was held at room temperature for one-half to one hour prior to reinoculation into embryos to eliminate false positive tests. The last time a positive recovery was made, the virus was identified by the hemagglutination test and, in some cases, by the serum-neutralization test. A titer of 80 or higher was the criterion for a positive hemagglutination test.

RESULTS

Table 1 gives a summary of the survival periods of the virus in the different materials stored at the various temperature conditions. The results of the two trials show that the maximum periods of virus viability on the different materials were determined at incubator, normal room, and hen house conditions of storage.

The results obtained under the five storage conditions show the same general trend. Burlap, filter paper, and cloth were test materials on which the virus was viable for the shortest periods at almost all conditions of storage. Eggshells, held at room temperature or higher, may also be classed with the latter test materials. The virus remained viable for the longest period in whole eggs and chick down at almost all storage conditions. At hen house and refrigerator temperatures, the virus diluted with broth or saline remained viable longer than when undiluted, while at the higher temperatures, the results were variable. Feces and broth were found to maintain virus viability for long intervals at the storage temperatures studied.

The virus in the undiluted amnioallantoic fluid stored in the deep freeze (–26 C.) maintained a fairly constant lethal titer for embryos throughout the duration of this study. The titer varied only by ten or one hundred fold. At the hen house and refrigerator conditions, there was a gradual decline in the titer of the virus until the final end point of viability was obtained. The period of decline was 199 days in the hen house and 258 days in the refrigerator. The titer of the virus stored at normal room temperature declined gradually until only the undiluted amnioallantoic fluid had a lethal effect on embryos. The period of decline was forty-two days in the first trial and seventy-one days in the second trial.

The undiluted amnioallantoic fluid stored in the refrigerator and deep freeze maintained a constant hemagglutination titer for 538 days, the duration of this study. The undiluted amnioallantoic fluid possessed a significant titer after fifteen months in the hen house, after twelve months at room temperature, and after forty-two days in the incubator.

The physical condition of the various test materials was altered after storage at temperatures above freezing. Cloth, filter paper, and burlap retained no perceptible moisture after two to twenty days' storage at room and incubator temperatures and after two to three months' storage at the hen house and refrigerator temperatures. Eggshells, soil, and feces remained moist much longer. Moderate evaporation occurred with the saline, broth, and amnioallantoic fluid. The amnioallantoic fluid also became thickened upon evaporation. In the case of whole eggs, the contents became thickened and caked (incubator, 40 to 60 days; normal room, 197 to 228 days).

DISCUSSION

Newcastle disease virus is equally or more resistant than several of the other viruses which may affect the respiratory tract of fowl. The duration of viability of the viruses causing laryngotracheitis, infectious bronchitis, and fowlpox has been reported by numerous investigators.[2,6,9,13,14]

Wooding[14] found that NDV was viable on chick feathers for at least sixty-three days at 37 C. In these experiments, the viability period of the virus on chick down was eighty-seven days. The viability results of virus seeded on filter paper are in agreement with those of Asplin.[1]

The viability of the virus was influenced by several factors, the most important of which was the temperature of storage—viability and temperature varying inversely. The freezing and thawing temperatures in the hen house may have influenced the duration of virus viability.

Drying was also important. The test materials (cloth, eggshells, filter paper, and burlap) which dried most rapidly were among the first to yield negative results. Mash, feces, and soil became dry only after prolonged storage and, at most conditions of storage, the virus remained viable in them longer than in the previously mentioned materials.

It is difficult to explain the reason for the variations in viability periods of the virus in broth, saline, and amnioallantoic fluid. Broth, at almost all conditions, was the most satisfactory of the three mediums, probably because of its protein content. At 37 C., the difference in the viability periods of the virus in amnioallantoic fluid, broth, and saline was not as extreme as at the lower temperatures of storage. At the lower temperatures, in most cases, diluted virus remained viable longer than undiluted virus.

The duration of viability of the virus may have been influenced by the general nature of the test materials. Those materials which may have afforded protection by forming a protein layer or film for the virus appeared to favor retention of the virus viability for longer periods. Materials which probably fall into this class are chick down, eggs, and broth.

Another factor which may have been influential in maintaining virus viability was the pH of the medium. The technical difficulties encountered in handling the various materials may have influenced the results obtained.

This study did not duplicate natural environmental conditions, and, for this reason, the wide range of test materials and conditions of storage were selected. Materials such as cloth, burlap, feces, mash, soil, and eggs, if contaminated with the virus in a natural environment, may be potential transmitters of the disease on the same premises, to other premises, and perhaps even from birds to human beings. It is quite likely, too, that the virus may survive in the natural environment from one season to the next.

This study emphasizes the need for sanitary practices in controlling the spread of this virus disease. Materials which may have been exposed to NDV should be well aërated and disinfected to insure against transmission of the virus.

SUMMARY

The virus of Newcastle disease when seeded on various test materials remained viable for long periods. The duration of viability of the virus was greatly influenced by the type of test material used. Among the materials tested, fresh eggs, chick down, and broth were the most suitable for maintaining virus viability.

The temperature of storage exerted an important influence on the duration of viability of the virus. The duration of viability tends to decrease at temperatures of 20 C. or higher, with a marked decrease at 37 C.

The virus was viable in fresh eggs for the following periods at the described conditions of storage: incubator, 126 days; normal room, 235 days; hen house, 255 days; refrigerator, 538 days (last test). On down, the virus was viable for the following periods: incubator, 87 days; normal room, 192 days; hen house, 255 days; refrigerator, 538 days; deep freeze, 538 days. The last two periods are not end points in viability but indicate the duration of time covered in this study.

The virus as contained in undiluted amnioallantoic fluid maintained a significant hemagglutination titer for the following storage periods: incubator, one month and twelve days; normal room, twelve months; hen house, fifteen months; refrigerator, eighteen months; deep freeze, eighteen months.

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The Effect of Aureomycin Against Salmonella Gallinarum in Vivo and in Vitro

J. E. PRIER, D.V.M., M.S., and J. O. ALBERTS, V.M.D., Ph.D.

Urbana, Illinois

LITTLE[1] REPORTED that experimental infections with *Salmonella gallinarum* in chicks were relatively resistant to aureomycin therapy. It required 36 doses of 1 mg. each at four-hour intervals to protect 70 per cent of infected day-old chicks. The present paper gives further experimental evidence on the *in vitro* and *in vivo* effects of aureomycin against *S. gallinarum*. Comparative *in vitro* tests were also run with penicillin and streptomycin.

MATERIALS AND METHODS

In Vitro Tests.—Two strains of *S. gallinarum* (10-4 and 10-5) isolated from outbreaks of fowl typhoid in Illinois poultry flocks were used. The organisms had been stored following lyophilization. Three mediums were used: tryptone, tryptose, and brain-heart infusion broths. Serial dilutions were made with each medium from 1,600 γ per milliliter through 0.7812 γ per ml. The final volume in each tube was 2 ml. All tubes, including a control tube, were inoculated with a loopful of a 24-hour brain-heart infusion broth culture of the organism. The end point of inhibition was considered to be the last tube in which no growth was visible. The tubes were examined twelve, twenty-four, forty-eight, seventy-two, and ninety-six hours after inoculation. The second tube from the last showing no growth at the end of ninety-six hours was diluted with five parts of broth and incubated for ninety-six hours to determine if the bacteria were alive. All tests were run in triplicate.

In Vivo Tests.—Chicks varying in age from 1 to 14 days were used. Infection was by intraperitoneal inoculation of 0.1 ml. of the 24-hour brain-heart infusion broth culture of *S. gallinarum*. Approximately, 13,000,000 organisms were contained in this inoculum.

Aureomycin hydrochloride was used in treatment. It was administered intraperitoneally or mixed in the feed. When the drug was given intraperitoneally, one or two injections of 5 or 10 mg. each were made. In some cases, the treatment was started twenty-four hours after infection and, in others, simultaneously with infection.

When the drug was mixed in the feed (1 mg./Gm. of feed), treatment was started either twenty-four hours before infection and continued for three to eight days after infection, or treatment was withheld until several birds had died and then given for seven or eight successive days.

TABLE I—In Vitro Effect of Aureomycin, Streptomycin, and Penicillin Against Salmonella Gallinarum

Strain	Medium	Antibiotic	End point of inhibition (γ/ml.)*				
			12(hr.)	24(hr.)	48(hr.)	72(hr.)	96(hr.)
10-4	BHI**	Aureomycin	8.30	33.30	100.00	200.00	.....
10-4	BHI	Streptomycin	200.00	266.00	333.00	400.00	.....
10-4	BHI	Penicillin	33.30	33.30	50.00	50.00	.....
10-4	Tryptone	Aureomycin	0.78	1.56	12.50	50.00	100.00
10-4	Tryptone	Streptomycin	6.25	12.50	33.30	41.70	41.70
10-4	Tryptone	Penicillin	12.50	12.50	16.70	16.70	16.70
10-4	Tryptose	Aureomycin	1.56	6.25	50.00	133.30	133.30
10-4	Tryptose	Streptomycin	50.00	66.70	133.30	133.30	133.30
10-4	Tryptose	Penicillin	12.50	12.50	12.50	12.50	12.50
10-5	BHI	Aureomycin	12.50	41.70	200.00	266.00	.....
10-5	BHI	Streptomycin	200.00	200.00	400.00	400.00	.....
10-5	BHI	Penicillin	41.70	50.00	50.00	50.00	.....
10-5	Tryptone	Aureomycin	0.78	1.56	12.50	50.00	100.00
10-5	Tryptone	Streptomycin	6.25	20.80	41.70	41.70	41.70
10-5	Tryptone	Penicillin	12.50	20.80	25.00	25.00	25.00
10-5	Tryptose	Aureomycin	12.50	41.70	400.00	1600.00	1600.00
10-5	Tryptose	Streptomycin	50.00	100.00	266.00	266.00	333.00
10-5	Tryptose	Penicillin	25.00	50.00	50.00	50.00	50.00

*Highest dilution showing completed inhibition growth. Average of three tests. **Brain-heart infusion broth.

From the College of Veterinary Medicine, University of Illinois, and the Agricultural Experiment Station, Urbana, Ill.

At present, Dr. Prier is head of the Department of Veterinary Science and Bacteriology, University of Wyoming, Laramie.

The technical assistance of Gerald Dart is acknowledged.

RESULTS

In Vitro Tests.—The results are shown in table 1. The initial inhibitory end point of aureomycin was greater than those of either streptomycin or penicillin. At seventy-two and ninety-six hours, however, this inhibition was markedly less, and penicillin produced a greater inhibition than either aureomycin or streptomycin. Streptomycin was the least inhibitory of the three antibiotics used. The initial inhibition (12 hr.) for aureomycin in brain-heart infusion broth was 8.3 and 12.5 γ/ml. At seventy-two hours, the inhibition end point was 200 and 266 γ/ml. Streptomycin in the same medium inhibited the organism from the initial point of 200 γ/ml. to 400 γ/ml. at seventy-two hours. Penicillin varied from 33.3 γ/ml. at twelve hours to 50 γ/ml. at seventy-two hours. In tryptone and tryptose broth, aureomycin inhibited the organisms in a range of 0.8 to 12.5 γ/ml. At ninety-six hours, the inhibition range was from 100 to 1,600 γ/ml. Streptomycin inhibited *S. gallinarum* in a range from 6.25 to 50 γ/ml. at twelve hours and from 41.7 to 333.0 γ/ml. at ninety-six hours. Penicillin varied from 12.5 to 25.0 γ/ml. at twelve hours to 12.5 to 50 γ/ml. at ninety-six hours.

Apparently brain-heart infusion broth was instrumental in reducing the activity of aureomycin and streptomycin. The effect was not noticeable with penicillin. Tryptone and tryptose broths differed little in their effects on the activity of the antibiotics.

TABLE 2—Effect of Aureomycin on Chicks Experimentally Infected with Salmonella Gallinarum

No. chicks	Age (da.)	Strain of organism*	Treatment	Survived (%)
20	4	10-5	10 mg. intraperitoneally 24 hours after infection	15.0
21	4	10-5	none	0.0
20	12	10-5	10 mg. intraperitoneally 24 hours after infection	50.0
16	12	10-5	none	37.0
5	12	none	10 mg. intraperitoneally	80.0
5	12	none	none	100.0
20	14	10-4	5 mg. intraperitoneally 24 hours after infection	45.0**
20	14	10-4	none	55.0
5	14	none	5 mg. intraperitoneally	80.0
5	14	none	none	100.0
20	1	10-4	5 mg. intraperitoneally at time of infection and 5 mg. 24 hours later	10.0
20	1	10-4	none	0.0
12	1	none	10 mg. in 2 equal doses 24 hours apart	58.0*
12	1	none	none	75.0
141	14	10-4	1 mg./Gm. of feed for 4 days beginning 24 hours before infection	59.5
203	14	10-4	none	2.0
56	14	none	1 mg./Gm. of feed for 4 days	98.3
201	6	10-5	1 mg./Gm. of feed for 8 days starting 3 days after infection	20.9
172	6	10-5	none	11.6
154	8	10-5	1 mg./Gm. of feed for 9 days starting 24 hours before infection	55.2
208	8	10-5	1 mg./Gm. of feed for 7 days starting 2 days after infection	40.4
202	8	10-5	none	5.5
50	8	none	1 mg./Gm. of feed for 9 days	100.0

*Infection by intraperitoneal inoculation of 0.1 ml. of 24-hour brain-heart infusion broth culture of *Salmonella gallinarum*. **No. pathogenic bacteria found on blood-agar plate cultures from livers of 4 birds.

In Vivd Tests.—The results are given in table 2. Intraperitoneal injections of 5 and 10 mg. of aureomycin twenty-four hours after exposure reduced losses 10 to 15 per cent. When 5 mg. of aureomycin were given at the time of exposure and 5 mg. twenty-four hours later, mortality was reduced 10 per cent in day-old chicks.

Birds that received aureomycin in the feed at the rate of 1 mg. per gram starting twenty-four hours before exposure and continuing three and eight days after exposure suffered losses of 40.5 and 44.8 per cent, while 98 and 94.5 per cent of the untreated control birds died. The same concentration of aureomycin in the feed was not as effective when started two and three days after exposure and continued for seven and eight days. Losses were 59.6 and 79.1 per cent, respectively, when therapy was delayed two and three days. The treated feed was apparently nontoxic for chicks.

SUMMARY

Intraperitoneal inoculations of 5 and 10 mg. of aureomycin were not effective in reducing losses in chicks experimentally infected with *Salmonella gallinarum*. Aureomycin given in the feed as a prophylactic

treatment for four and nine days, starting twenty-four hours before infection, reduced losses to 40.5 and 44.8 per cent, respectively, while 98 and 94.5 per cent of the untreated controls succumbed. Delay of treatment for two and three days after exposure was less effective, since the mortality was 59.6 and 79.1 per cent, respectively.

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New Molluscicides for the Control of Fresh Water Snails

EDWARD G. BATTE, D.V.M., M.S.; LEONARD E. SWANSON, D.V.M.; J. B. MURPHY, B.S.

Gainesville, Florida

EVERY FLUKE which infects man or domestic animals utilizes a snail as a necessary intermediate host in order to complete its life cycle. The logical point of attack in the control of fluke diseases is an efficient and practical method of destroying the snails which serve as their intermediate hosts. Where it is possible from the economic and mechanical viewpoints, the complete drainage of snail habitats will insure permanent control. In some areas, this is impossible and other methods of snail control must be employed.

LITERATURE

Thomas[1] suggested the application of sodium chloride on pastures for control of snails that serve as intermediate hosts of *Fasciola hepatica*, Linn. Leiper[2] recommended that the flow of water in irrigation ditches be stopped for fifteen-day periods, thinking that this procedure would destroy the snails by desiccation. He further advocated the application of ammonium sulfate to pools for the control of snails which serve as intermediate hosts of Schistosoma. Ando[3] observed that 1 per cent ammonium sulfate killed 6 of 10 snails in seven hours, and 1 per cent copper sulfate killed all of the snails in six hours. Chandler[4] reported that, with equivalent concentrations of the copper ion, the toxicity of the various copper salts was approximately the same. Copper sulfate was selected for further experimentation because it was the cheapest copper salt. Halawani[5] found that benzene hexachloride was toxic to two genera of snails (Planorbis and Bulinus) which are intermediate hosts of Schistosoma in Egypt.

Schreiber and Schubert[6] reported that quinones and tertiary amines were most toxic to schistosome cercariae. Jachowski and Stirewalt[7] found that the *alpha*-chloro-esters and amides of aliphatic monobasic acids showed a more marked molluscicidal activity than unchlorinated derivatives to *Australorbis glabratus* (Say). McMullen and Graham[8] screened numerous chemicals in an effort to find a satisfactory control for *Oncomelania quadrasi* Moll., which is the molluscan intermediate host for schistosomiasis in the Philippines. They found that dinitro-o-cyclohexylphenol and its dicyclohexylamine salt gave the best results. McMullen and Ingalls[9] reported that these compounds had a good residual effect and would kill fluke cercariae as well as the snails. Kuntz and Stirewalt[10] observed that, at concentrations as low as one-half part per million of dinitro-o-cyclohexylphenol, snail embryos failed to hatch.

PROCEDURE

In screening new chemicals for possible use as molluscicides, stock solutions were made with distilled water so as to contain 1 per cent, and in turn 0.1 per cent, of active ingredients. Dilutions were made from the 0.1 per cent solution into 1,000-cc. beakers containing enough ditch water to obtain a total volume of 500 cc. An even distribution was obtained by agitation. Five lymnaeid snails, which serve as intermediate hosts for liver flukes in Flor-

From the Department of Veterinary Science, Agricultural Experiment Station, University of Florida, Gainesville.

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ida, were added to each of these beakers, one beaker for each dilution and one for a control. Observations were made after two, four, eight, and twenty-four hours, and the results were recorded as to whether the snails were alive or dead. In these tests, a total of 33 compounds were used in concentrations varying from 1 : 100,000 to 1 : 1,000,000. Results are shown in table 1. There was no mortality of any snail used as a control.

RESULTS

An arbitrary standard might be set for an ideal molluscicide. It should kill 100 per cent of the test snails within twenty-four hours at a dilution of 1 : 1,000,000. It should be nontoxic to man, grass, and animals which might drink the treated water or eat the plants. The compound should be relatively inexpensive and should not be affected by the organic content of the water. Snail embryos should be killed on contact by the compound, or its residual action should be long enough to kill young snails soon after hatching.

Of the 33 compounds tested, only dinitro-o-cyclohexylphenol and copper sulfate gave 100 per cent kill of lymnaeid snails in a dilution of 1 : 1,000,000 in twenty-four hours. Crude benzene hexachloride and sodium tetrachlorophenate gave 100 per cent kill in a dilution of 1 : 600,000. There was so much difference between the kill of crude 5 per cent benzene hexachloride and pure *gamma* isomer that it is believed that some of the other isomers might prove to be good molluscicides. The pure isomers, *alpha*, *beta*, and *delta*, alone and in combination, failed to give any kill at 1 : 100,000. Later information revealed that the crude benzene hexachloride contained 35 per cent *alpha* isomer, 5 per cent *gamma* isomer, 5 per cent *beta* isomer, and 5 per cent *delta* and other isomers. Based upon a dilution of 1 : 100,000 of the *gamma* isomer, there was a concentration of 1 : 15,000 of the *alpha* isomer, which may have accounted for the toxicity.

Other compounds in the dinitro and chlorophenate series are being tested for their

TABLE I—Toxicity of Some Organic Compounds to Lymnaeid Snails Following Twenty-Four-Hour Exposure

A. 100 per cent mortality in concentrations of 1 : 1,000,000 and greater
   1. Dinitro-o-cyclohexylphenol (DN No. 1) 40 per cent
   2. Cupric sulfate, anhydrous

B. 100 per cent mortality in concentrations of 1 : 600,000 and greater
   1. Crude benzene hexachloride, *gamma* isomer 5 per cent
   2. Sodium 2, 3, 4, 6-tetrachlorophenate (dowicide F) 80 per cent

C. 100 per cent mortality in concentrations of 1 : 500,000 and greater
   1. Dinitro-o-cyclohexylphenol, dicyclohexylamine salt (DN-111) 20 per cent

D. 100 per cent mortality in concentrations of 1 : 400,000 and greater
   1. Sodium pentachlorophenate, 88 per cent
   2. Hexachloro-epoxyoctahydro-dimethanonaphthalene (dieldrin) 25 per cent
   3. O, o, diethyl-o-p-nitrophenyl thiophosphate (parathion) 15 per cent
   4. 2, 4-dinitrothymol, tech
   5. 4 chlorohexyl-2, 6-dinitrophenol, tech
   6. Dinitro-o-cyclohexylphenol, 38 per cent

E. 100 per cent mortality in concentrations of 1 : 200,000 and greater
   1. Dinitro-o-cyclohexylphenol, dicyclohexylamine salt (K604) tech
   2. Dialkyl nitroaryl thiophosphate (metacide) 33.4 per cent
   3. Dinitro-o-sec butylphenol, tech
   4. Chloro-2-phenyl phenol (dowicide 31) tech

F. 100 per cent mortality in concentrations of 1 : 100,000 and greater
   1. Hexachlorohexahydro-dimethanonaphthalene (aldrin) 48 per cent
   2. Benzene hexachloride, 99.8 per cent *gamma* isomer

G. Not 100 per cent mortality in concentrations of 1 : 100,000
   1. Benzene hexachloride
      a. *Alpha* isomer, tech
      b. *Beta* isomer, tech
      c. *Delta* isomer, tech
      d. *Alpha-beta* isomer, tech
      e. *Alpha-delta* isomer, tech
      f. *Beta-delta* isomer, tech
      g. *Alpha-beta-delta* isomer, tech
   2. Ethyl para nitrophenyl thionobenzenephosphonate (EPN miticide) 31.5 per cent
   3. M-dinitro-benzene, tech
   4. 2,6-dinitro-4-chlorophenol, tech
   5. 3,5-dinitro-o-cresol, tech
   6. 2,4-dinitro-o-cresol, tech
   7. 4,6-dinitro-o-cresol acetate, tech
   8. 4,6-dinitro-o-cresol methyl ether, tech
   9. 2,4-dinitrophenol, tech
   10. 2,4-dinitrophenol, sodium salt, tech
   11. 2,4-dinitrophenolhydrazine, tech
   12. 2,4-dinitrotoluene, tech
   13. 0,0-dimethyl dithiophosphate (4049) 47.7 per cent
   14. *Beta*-chloroethyl, *beta*-(P-tertiarybutylphenoxy) *alpha*-methylethyl sulfite (aramite) 15 per cent

molluscicidal activity. Also, some of the more promising compounds are being tested for their killing power against snail ova, fluke ova, miracidia, and metacercariae.

SUMMARY

A total of 33 compounds was tested for molluscicidal activity against lymnaeid snails; however, only copper sulfate and dinitro-o-cyclohexylphenol killed 100 per cent of the test snails at a concentration of 1 : 1,000,000 in twenty-four hours. Other compounds in the dinitro and chlorophenate series have shown promise and will be tested and compared with copper sulfate.

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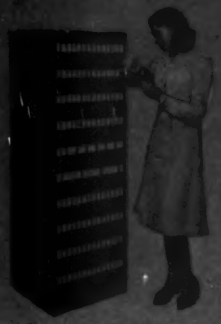

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